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(54) Title: METHOD AND APPARATUS FOR IDENTIFYING, CLASSIFYING, OR QUANTIFYING DNA SEQUENCES IN A SAMPLE WITHOUT SEQUENCING			
(57) Abstract <p>This invention provides methods by which biologically derived DNA sequences in a mixed sample or in an arrayed single sequence clone can be determined and classified without sequencing. The methods make use of information on the presence of carefully chosen target subsequences, typically of length from 4 to 8 base pairs, and preferably the length between target subsequences in a sample DNA sequence together with DNA sequence databases containing lists of sequences likely to be present in the sample to determine a sample sequence. The preferred method uses restriction endonucleases to recognize target subsequences and cut the sample sequence. Then carefully chosen recognition moieties are ligated to the cut fragments, the fragments amplified, and the experimental observation made. Polymerase chain reaction (PCR) is the preferred method of amplification. Several alternative embodiments are described which are capable of increased discrimination and which use Type IIIS restriction endonucleases, various capture moieties, or samples of specially synthesized cDNA. Another embodiment of the invention uses information on the presence or absence of carefully chosen target subsequences in a single sequence clone together with DNA sequence databases to determine the clone sequence. Computer implemented methods are provided to analyze the experimental results and to determine the sample sequences in question and to carefully choose target subsequences in order that experiments yield a maximum amount of information.</p>			

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METHOD AND APPARATUS FOR IDENTIFYING,
CLASSIFYING, OR QUANTIFYING DNA SEQUENCES
IN A SAMPLE WITHOUT SEQUENCING

The application is a continuation-in-part of
5 copending U.S. Patent Application serial number 08/547,214,
filed on October 24, 1995, which is hereby incorporated by
reference in its entirety.

This invention was made with United States
Government support under award number 70NANB5H1036 awarded by
10 the National Institute of Standards and Technology. The
United States Government has certain rights in the invention.

1. FIELD OF THE INVENTION

The field of this invention is DNA sequence
15 classification, identification or determination, and
quantification; more particularly it is the quantitative
classification, comparison of expression, or identification
of preferably all DNA sequences or genes in a sample without
performing any sequencing.

20

2. BACKGROUND

Over the past ten years, as biological and genomic
research have revolutionized our understanding of the
molecular basis of life, it has become increasingly clear
25 that the temporal and spatial expression of genes is
responsible for all life's processes, processes occurring in
both health and in disease. Science has progressed from an
understanding of how single genetic defects cause the
traditionally recognized hereditary disorders, such as the
30 thalassemias, to a realization of the importance of the
interaction of multiple genetic defects along with
environmental factors in the etiology of the majority of more
complex disorders, such as cancer. In the case of cancer,
current scientific evidence demonstrates the key causative
35 roles of altered expression of and multiple defects in
several pivotal genes. Other complex diseases have similar

etiology. Thus the more complete and reliable a correlation that can be established between gene expression and health or disease states, the better diseases can be recognized, diagnosed and treated.

5 This important correlation is established by the quantitative determination and classification of DNA expression in tissue samples, and such a method which is rapid and economical would be of considerable value. Genomic DNA ("gDNA") sequences are those naturally occurring DNA 10 sequences constituting the genome of a cell. The state of gene, or gDNA, expression at any time is represented by the composition of total cellular messenger RNA ("mRNA"), which is synthesized by the regulated transcription of gDNA. Complementary DNA ("cDNA") sequences are synthesized by 15 reverse transcription from mRNA. cDNA from total cellular mRNA also represents, albeit approximately, gDNA expression in a cell at a given time. Consequently, rapid and economical detection of all the DNA sequences in particular cDNA or gDNA samples is desired, particularly so if such 20 detection was rapid, precise, and quantitative.

Heretofore, gene specific DNA analysis techniques have not been directed to the determination or classification of substantially all genes in a DNA sample representing total cellular mRNA and have required some degree of sequencing. 25 Generally, existing cDNA, and also gDNA, analysis techniques have been directed to the determination and analysis of one or two known or unknown genetic sequences at one time. These techniques have used probes synthesized to specifically recognize by hybridization only one particular DNA sequence 30 or gene. (See, e.g., Watson et al., 1992, Recombinant DNA, chap 7, W. H. Freeman, New York.) Further, adaptation of these methods to the problem of recognizing all sequences in a sample would be cumbersome and uneconomical.

One existing method for finding and sequencing 35 unknown genes starts from an arrayed cDNA library. From a particular tissue or specimen, mRNA is isolated and cloned into an appropriate vector, which is then plated in a manner

so that the progeny of individual vectors bearing the clone of one cDNA sequence can be separately identified. A replica of such a plate is then probed, often with a labeled DNA oligomer selected to hybridize with the cDNA representing the 5 gene of interest. Thereby, those colonies bearing the cDNA of interest are found and isolated, the cDNA harvested and subject to sequencing. Sequencing can then be done by the Sanger dideoxy chain termination method (Sanger et al., 1977, "DNA sequencing with chain terminating inhibitors", Proc. 10 Natl. Acad. Sci. USA 74(12):5463-5467) applied to inserts so isolated.

The DNA oligomer probes for the unknown gene used for colony selection are synthesized to hybridize, preferably, only with the cDNA for the gene of interest. One 15 manner of achieving this specificity is to start with the protein product of the gene of interest. If a partial sequence of 5 to 10-mer peptide fragment from an active region of this protein can be determined, corresponding 15 to 30-mer degenerate oligonucleotides can be synthesized which 20 code for this peptide. This collection of degenerate oligonucleotides will typically be sufficient to uniquely identify the corresponding gene. Similarly, any information leading to 15 to 30 long nucleotide subsequences can be used to create a single gene probe.

25 Another existing method, which searches for a known gene in a cDNA or gDNA prepared from a tissue sample, also uses single gene or single sequence probes which are complementary to unique subsequences of the already known gene sequences. For example, the expression of a particular 30 oncogene in sample can be determined by probing tissue derived cDNA with a probe derived from a subsequence of the oncogene's expressed sequence tag. Similarly the presence of a rare or difficult to culture pathogen, such as the TB bacillus or the HIV, can be determined by probing gDNA with a 35 hybridization probe specific to a gene of the pathogen. The heterozygous presence of a mutant allele in a phenotypically normal individual, or its homozygous presence in a fetus, can

be determined by probing with an allele specific probe complementary only to the mutant allele (See, e.g., Guo et al., 1994, *Nucleic Acid Research*, 22:5456-65).

All existing methods using single gene probes, of which the preceding examples are typical, if applied to determine all genes expressed in a given tissue sample, would require many thousands to tens of thousands of individual probes. It is estimated a single human cell typically expresses approximately to 15,000 to 15,000 genes simultaneously and that the most complex tissue, e.g., the brain, can express up to half the human genome (Liang et al., 1992, "Differential Display of Eukaryotic Messenger RNA by Means of the Polymerase Chain Reaction, *Science*, 257:967-971). Such an application requiring such a number of probes is clearly too cumbersome to be economic or, even, practical.

Another class of existing methods, known as sequencing by hybridization ("SBH"), in contrast, use combinatorial probes which are not gene specific (Drmanac et al., 1993, *Science* 260:1649-52; U.S. Patent No. 5,202,231, 20 Apr 13, 1993, to Drmanac et al.). An exemplary implementation of SBH to determine an unknown gene requires that a single cDNA clone be probed with all DNA oligomers of a given length, say, for example, all 6-mers. Such a set of all oligomers of a given length synthesized without any selection is called a combinatorial probe library. From knowledge of all hybridization results for a combinatorial library, say all the 4096 6-mer probe results, a partial DNA sequence for the cDNA clone can be reconstructed by algorithmic manipulations. Complete sequences are not determinable because, at least, repeated subsequences cannot be fully determined. SBH adapted to the classification of known genes is called oligomer sequence signatures ("OSS") (Lennon et al., 1991, *Trends In Genetics* 7(10):314-317). This technique classifies a single clone based on the pattern of probe hits against an entire combinatorial library, or a significant sub-library. It requires that the tissue sample library be

arrayed into clones, each clone comprising only one pure sequence from the library. It cannot be applied to mixtures.

These exemplary existing methods are all directed to finding one sequence in an array of clones each expressing 5 a single sequence from a tissue sample. They are not directed to rapid, economical, quantitative, and precise characterization of all the DNA sequences in a mixture of sequences, such as a particular total cellular cDNA or gDNA sample. Their adaptation to such a task would be 10 prohibitive. Determination by sequencing the DNA of a clone, much less an entire sample of thousands of sequences, is not rapid or inexpensive enough for economical and useful diagnostics. Existing probe-based techniques of gene determination or classification, whether the genes are known 15 or unknown, require many thousands of probes, each specific to one possible gene to be observed, or at least thousands or even tens of thousands of probes in a combinatorial library. Further, all of these methods require the sample be arrayed into clones each expressing a single gene of the sample.

20 In contrast to the prior exemplary existing gene determination and classification techniques, another existing technique, known as differential display, attempts to fingerprint a mixture of expressed genes, as is found in a pooled cDNA library. This fingerprint, however, seeks merely 25 to establish whether two samples are the same or different.

No attempt is made to determine the quantitative, or even qualitative, expression of particular, determined genes (Liang et al., 1995, *Current Opinions in Immunology* 7:274-280; Liang et al., 1992, *Science* 257:967-71; Welsh et al., 30 1992, *Nucleic Acid Res.* 20:4965-70; McClelland et al., 1993, *Exs* 67:103-15; Lisitsyn, 1993, *Science* 259:946-50).

Differential display uses the polymerase chain reaction ("PCR") to amplify DNA subsequences of various lengths, which are defined by being between the hybridization sites of 35 arbitrarily selected primers. Ideally, the pattern of lengths observed is characteristic of the tissue from which the library was prepared. Typically, one primer used in

differential display is oligo(dT) and the other is one or more arbitrary oligonucleotides designed to hybridize within a few hundred base pairs of the poly-dA tail of a cDNA in the library. Thereby, on electrophoretic separation, the 5 amplified fragments of lengths up to a few hundred base pairs should generate bands characteristic and distinctive of the sample. Changes in tissue gene expression may be observed as changes in one or more bands.

Although characteristic banding patterns develop, 10 no attempt is made to link these patterns to the expression of particular genes. The second arbitrary primer cannot be traced to a particular gene. First, the PCR process is less than ideally specific. One to a few base pair ("bp") mismatches ("bubbles") are permitted by the lower stringency 15 annealing step typically used and are tolerated well enough so that a new chain can be initiated by the *Taq* polymerase, often used in PCR reactions. Second, the location of a single subsequence or its absence is insufficient information to distinguish all expressed genes. Third, length 20 information from the arbitrary primer to the poly-dA tail is generally not found to be characteristic of a sequence due to variations in the processing of the 3' untranslated regions of genes, the variation in the poly-adenylation process and variability in priming to the repetitive sequence at a 25 precise point. Thus, even the bands that are produced often are smeared by the non-specific background sequences present. Also known PCR biases to high G+C content and short sequences further limit the specificity of this method. Thus this technique is generally limited to "fingerprinting" samples 30 for a similarity or dissimilarity determination and is precluded from use in quantitative determination of the differential expression of identifiable genes.

Existing methods for gene or DNA sequence classification or determination are in need of improvement in 35 their ability to perform rapid and economical as well as quantitative and specific determination of the components of a cDNA mixture prepared from a tissue sample. The preceding

background review identifies the deficiencies of several exemplary existing methods.

3. SUMMARY OF THE INVENTION

5 It is an object of this invention to provide methods for rapid, economical, quantitative, and precise determination or classification of DNA sequences, in particular genomic or complementary DNA sequences, in either arrays of single sequence clones or mixtures of sequences 10 such as can be derived from tissue samples, without actually sequencing the DNA. Thereby, the deficiencies in the background arts just identified are solved. This object is realized by generating a plurality of distinctive and detectable signals from the DNA sequences in the sample being 15 analyzed. Preferably, all the signals taken together have sufficient discrimination and resolution so that each particular DNA sequence in a sample may be individually classified by the particular signals it generates, and with reference to a database of DNA sequences possible in the 20 sample, individually determined. The intensity of the signals indicative of a particular DNA sequence depends quantitatively on the amount of that DNA present. Alternatively, the signals together can classify a predominant fraction of the DNA sequences into a plurality of 25 sets of approximately no more than two to four individual sequences.

It is a further object that the numerous signals be generated from measurements of the results of as few a number of recognition reactions as possible, preferably no more than 30 approximately 5-400 reactions, and most preferably no more than approximately 20-50 reactions. Rapid and economical determinations would not be achieved if each DNA sequence in a sample containing a complex mixture required a separate reaction with a unique probe. Preferably, each recognition 35 reaction generates a large number of or a distinctive pattern of distinguishable signals, which are quantitatively proportional to the amount of the particular DNA sequences

present. Further, the signals are preferably detected and measured with a minimum number of observations, which are preferably capable of simultaneous performance.

The signals are preferably optical, generated by 5 fluorochrome labels and detected by automated optical detection technologies. Using these methods, multiple individually labeled moieties can be discriminated even though they are in the same filter spot or gel band. This permits multiplexing reactions and parallelizing signal 10 detection. Alternatively, the invention is easily adaptable to other labeling systems, for example, silver staining of gels. In particular, any single molecule detection system, whether optical or by some other technology such as scanning or tunneling microscopy, would be highly advantageous for use 15 according to this invention as it would greatly improve quantitative characteristics.

According to this invention, signals are generated by detecting the presence (hereinafter called "hits") or absence of short DNA subsequences (hereinafter called 20 "target" subsequences) within a nucleic acid sequence of the sample to be analyzed. The presence or absence of a subsequence is detected by use of recognition means, or probes, for the subsequence. The subsequences are recognized by recognition means of several sorts, including but not 25 limited to restriction endonucleases ("REs"), DNA oligomers, and PNA oligomers. REs recognize their specific subsequences by cleavage thereof; DNA and PNA oligomers recognize their specific subsequences by hybridization methods. The preferred embodiment detects not only the presence of pairs 30 of hits in a sample sequence but also include a representation of the length in base pairs between adjacent hits. This length representation can be corrected to true physical length in base pairs upon removing experimental biases and errors of the length separation and detection 35 means. An alternative embodiment detects only the pattern of hits in an array of clones, each containing a single sequence ("single sequence clones").

The generated signals are then analyzed together with DNA sequence information stored in sequence databases in computer implemented experimental analysis methods of this invention to identify individual genes and their quantitative 5 presence in the sample.

The target subsequences are chosen by further computer implemented experimental design methods of this invention such that their presence or absence and their relative distances when present yield a maximum amount of 10 information for classifying or determining the DNA sequences to be analyzed. Thereby it is possible to have orders of magnitude fewer probes than there are DNA sequences to be analyzed, and it is further possible to have considerably fewer probes than would be present in combinatorial libraries 15 of the same length as the probes used in this invention. For each embodiment, target subsequences have a preferred probability of occurrence in a sequence, typically between 5% and 50%. In all embodiments, it is preferred that the presence of one probe in a DNA sequence to be analyzed is 20 independent of the presence of any other probe.

Preferably, target subsequences are chosen based on information in relevant DNA sequence databases that characterize the sample. A minimum number of target subsequences may be chosen to determine the expression of all 25 genes in a tissue sample ("tissue mode"). Alternatively, a smaller number of target subsequences may be chosen to quantitatively classify or determine only one or a few sequences of genes of interest, for example oncogenes, tumor suppressor genes, growth factors, cell cycle genes, 30 cytoskeletal genes, etc ("query mode").

A preferred embodiment of the invention, named quantitative expression analysis ("QEA™"), produces signals comprising target subsequence presence and a representation of the length in base pairs along a gene between adjacent 35 target subsequences by measuring the results of recognition reactions on cDNA (or gDNA) mixtures. Of great importance, this method does not require the cDNA be inserted into a

vector to create individual clones in a library. Creation of these libraries is time consuming, costly, and introduces bias into the process, as it requires the cDNA in the vector to be transformed into bacteria, the bacteria arrayed as 5 clonal colonies, and finally the growth of the individual transformed colonies.

Three exemplary experimental methods are described herein for performing QEA™: a preferred method utilizing a novel RE/ligase/amplification procedure; a PCR based method; 10 and a method utilizing a removal means, preferably biotin, for removal of unwanted DNA fragments. The preferred method generates precise, reproducible, noise free signatures for determining individual gene expression from DNA in mixtures or libraries and is uniquely adaptable to automation, since 15 it does not require intermediate extractions or buffer exchanges. A computer implemented gene calling step uses the hit and length information measured in conjunction with a database of DNA sequences to determine which genes are present in the sample and the relative levels of expression. 20 Signal intensities are used to determine relative amounts of sequences in the sample. Computer implemented design methods optimize the choice of the target subsequences.

A second specific embodiment of the invention, termed colony calling ("CC"), gathers only target subsequence 25 presence information for all target subsequences for arrayed, individual single sequence clones in a library, with cDNA libraries being preferred. The target subsequences are carefully chosen according to computer implemented design methods of this invention to have a maximum information 30 content and to be minimum in number. Preferably from 10-20 subsequences are sufficient to characterize the expressed cDNA in a tissue. In order to increase the specificity and reliability of hybridization to the typically short DNA subsequences, preferable recognition means are PNAs. 35 Degenerate sets of longer DNA oligomers having a common, short, shared, target sequence can also be used as a recognition means. A computer implemented gene calling step

uses the pattern of hits in conjunction with a database of DNA sequences to determine which genes are present in the sample and the relative levels of expression.

The embodiments of this invention preferably 5 generate measurements that are precise, reproducible, and free of noise. Measurement noise in QEA™ is typically created by generation or amplification of unwanted DNA fragments, and special steps are preferably taken to avoid any such unwanted fragments. Measurement noise in colony 10 calling is typically created by mis-hybridization of probes, or recognition means, to colonies. High stringency reaction conditions and DNA mimics with increased hybridization specificity may be used to minimize this noise. DNA mimics are polymers composed of subunits capable of specific, 15 Watson-Crick-like hybridization with DNA. Also useful to minimize noise in colony calling are improved hybridization detection methods. Instead of the conventional detection methods based on probe labeling with fluorochromes, new methods are based on light scattering by small 100-200 μm 20 particles that are aggregated upon probe hybridization (Stimson et al., 1995, "Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides", Proc. Natl. Acad. Sci. USA 92:6379- 6383). In this method, the hybridization surface forms one 25 surface of a light pipe or optical wave guide, and the scattering induced by these aggregated particles causes light to leak from the light pipe. In this manner hybridization is revealed as an illuminated spot of leaking light on a dark background. This latter method makes hybridization detection 30 more rapid by eliminating the need for a washing step between the hybridization and detection steps. Further by using variously sized and shaped particles with different light scattering properties, multiple probe hybridizations can be detected from one colony.

35 Further, the embodiments of the invention can be adapted to automation by eliminating non-automatable steps, such as extractions or buffer exchanges. The embodiments of

the invention facilitate efficient analysis by permitting multiple recognition means to be tested in one reaction and by utilizing multiple, distinguishable labeling of the recognition means, so that signals may be simultaneously detected and measured. Preferably, for QEA™ embodiments, this labeling is by multiple fluorochromes. For the CC embodiments, detection is preferably done by the light scattering methods with variously sized and shaped particles.

An increase in sensitivity as well as an increase in the number of resolvable fluorescent labels can be achieved by the use of fluorescent, energy transfer, dye-labeled primers. Other detection methods, preferable when the genes being identified will be physically isolated from the gel for later sequencing or use as experimental probes, include the use of silver staining gels or of radioactive labeling. Since these methods do not allow for multiple samples to be run in a single lane, they are less preferable when high throughput is needed.

Because this invention achieves rapid and economical determination of quantitative gene expression in tissue or other samples, it has considerable medical and research utility. In medicine, as more and more diseases are recognized to have important genetic components to their etiology and development, it is becoming increasingly useful to be able to assay the genetic makeup and expression of a tissue sample. For example, the presence and expression of certain genes or their particular alleles are prognostic or risk factors for disease (including disorders). Several examples of such diseases are found among the neurodegenerative diseases, such as Huntington's disease and ataxia-telangiectasia. Several cancers, such as neuroblastoma, can now be linked to specific genetic defects. Finally, gene expression can also determine the presence and classification of those foreign pathogens that are difficult or impossible to culture *in vitro* but which nevertheless express their own unique genes.

Disease progression is reflected in changes in genetic expression of an affected tissue. For example, expression of particular tumor promoter genes and lack of expression of particular tumor suppressor genes is now known 5 to correlate with the progression of certain tumors from normal tissue, to hyperplasia, to cancer *in situ*, and to metastatic cancer. Return of a cell population to a normal pattern of gene expression, such as by using anti-sense technology, can correlate with tumor regression. Therefore, 10 knowledge of gene expression in a cancerous tissue can assist in staging and classifying this disease.

Expression information can also be used to chose and guide therapy. Accurate disease classification and staging or grading using gene expression information can 15 assist in choosing initial therapies that are increasingly more precisely tailored to the precise disease process occurring in the particular patient. Gene expression information can then track disease progression or regression, and such information can assist in monitoring the success or 20 changing the course of an initial therapy. A therapy is favored that results in a regression towards normal of an abnormal pattern of gene expression in an individual, while therapy which has little effect on gene expression or its progression can need modification. Such monitoring is now 25 useful for cancers and will become useful for an increasing number of other diseases, such as diabetes and obesity. Finally, in the case of direct gene therapy, expression analysis directly monitors the success of treatment.

In biological research, rapid and economical assay 30 for gene expression in tissue or other samples has numerous applications. Such applications include, but are not limited to, for example, in pathology examining tissue specific genetic response to disease, in embryology determining developmental changes in gene expression, in pharmacology 35 assessing direct and indirect effects of drugs on gene expression. In these applications, this invention can be applied, e.g., to *in vitro* cell populations or cell lines, to

in vivo animal models of disease or other processes, to human samples, to purified cell populations perhaps drawn from actual wild-type occurrences, and to tissue samples containing mixed cell populations. The cell or tissue 5 sources can advantageously be a plant, a single celled animal, a multicellular animal, a bacterium, a virus, a fungus, or a yeast, etc. The animal can advantageously be laboratory animals used in research, such as mice engineered or bred to have certain genomes or disease conditions or 10 tendencies. The in vitro cell populations or cell lines can be exposed to various exogenous factors to determine the effect of such factors on gene expression. Further, since an unknown signal pattern is indicative of an as yet unknown gene, this invention has important use for the discovery of 15 new genes. In medical research, by way of further example, use of the methods of this invention allow correlating gene expression with the presence and progress of a disease and thereby provide new methods of diagnosis and new avenues of therapy which seek to directly alter gene expression.

20 This invention includes various embodiments and aspects, several of which are described below.

In a first embodiment, the invention provides a method for identifying, classifying, or quantifying one or more nucleic acids in a sample comprising a plurality of 25 nucleic acids having different nucleotide sequences, said method comprising probing said sample with one or more recognition means, each recognition means recognizing a different target nucleotide subsequence or a different set of target nucleotide subsequences; generating one or more 30 signals from said sample probed by said recognition means, each generated signal arising from a nucleic acid in said sample and comprising a representation of (i) the length between occurrences of target subsequences in said nucleic acid and (ii) the identities of said target subsequences in 35 said nucleic acid or the identities of said sets of target subsequences among which is included the target subsequences in said nucleic acid; and searching a nucleotide sequence

database to determine sequences that match or the absence of any sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences of nucleic acids that may be present in the sample, 5 a sequence from said database matching a generated signal when the sequence from said database has both (i) the same length between occurrences of target subsequences as is represented by the generated signal and (ii) the same target subsequences as is represented by the generated signal, or 10 target subsequences that are members of the same sets of target subsequences represented by the generated signal, whereby said one or more nucleic acids in said sample are identified, classified, or quantified.

This invention further provides in the first 15 embodiment additional methods wherein each recognition means recognizes one target subsequence, and wherein a sequence from said database matches a generated signal when the sequence from said database has both the same length between occurrences of target subsequences as is represented by the 20 generated signal and the same target subsequences as represented by the generated signal, or optionally wherein each recognition means recognizes a set of target subsequences, and wherein a sequence from said database matches a generated signal when the sequence from said 25 database has both the same length between occurrences of target subsequences as is represented by the generated signal, and target subsequences that are members of the sets of target subsequences represented by the generated signal.

This invention further provides in the first 30 embodiment additional methods further comprising dividing said sample of nucleic acids into a plurality of portions and performing the methods of this object individually on a plurality of said portions, wherein a different one or more recognition means are used with each portion.

35 This invention further provides in the first embodiment additional methods wherein the quantitative abundance of a nucleic acid comprising a particular

nucleotide sequence in the sample is determined from the quantitative level of the one or more signals generated by said nucleic acid that are determined to match said particular nucleotide sequence.

5 This invention further provides in the first embodiment additional methods wherein said plurality of nucleic acids are DNA, and optionally wherein the DNA is cDNA, and optionally wherein the cDNA is prepared from a plant, a single celled animal, a multicellular animal, a 10 bacterium, a virus, a fungus, or a yeast, and optionally wherein the cDNA is of total cellular RNA or total cellular poly(A) RNA.

This invention further provides in the first embodiment additional methods wherein said database comprises 15 substantially all the known expressed sequences of said plant, single celled animal, multicellular animal, bacterium, or yeast.

This invention further provides in the first embodiment additional methods wherein the recognition means 20 are one or more restriction endonucleases whose recognition sites are said target subsequences, and wherein the step of probing comprises digesting said sample with said one or more restriction endonucleases into fragments and ligating double stranded adapter DNA molecules to said fragments to produce 25 ligated fragments, each said adapter DNA molecule comprising (i) a shorter stand having no 5' terminal phosphates and consisting of a first and second portion, said first portion at the 5' end of the shorter strand being complementary to the overhang produced by one of said restriction 30 endonucleases and (ii) a longer strand having a 3' end subsequence complementary to said second portion of the shorter strand; and wherein the step of generating further comprises melting the shorter strand from the ligated fragments, contacting the sample with a DNA polymerase, 35 extending the ligated fragments by synthesis with the DNA polymerase to produce blunt-ended double stranded DNA fragments, and amplifying the blunt-ended fragments by a

method comprising contacting said blunt-ended fragments with a DNA polymerase and primer oligodeoxynucleotides, said primer oligodeoxynucleotides comprising the longer adapter strand, and said contacting being at a temperature not 5 greater than the melting temperature of the primer oligodeoxynucleotide from a strand of the blunt-ended fragments complementary to the primer oligodeoxynucleotide and not less than the melting temperature of the shorter strand of the adapter nucleic acid from the blunt-ended 10 fragments.

This invention further provides in the first embodiment additional methods wherein the recognition means are one or more restriction endonucleases whose recognition sites are said target subsequences, and wherein the step of 15 probing further comprises digesting the sample with said one or more restriction endonucleases.

This invention further provides in the first embodiment additional methods further comprising identifying a fragment of a nucleic acid in the sample which generates 20 said one or more signals; and recovering said fragment, and optionally wherein the signals generated by said recovered fragment do not match a sequence in said nucleotide sequence database, and optionally further comprising using at least a hybridizable portion of said fragment as a hybridization 25 probe to bind to a nucleic acid that can generate said fragment upon digestion by said one or more restriction endonucleases.

This invention further provides in the first embodiment additional methods wherein the step of generating 30 further comprises after said digesting removing from the sample both nucleic acids which have not been digested and nucleic acid fragments resulting from digestion at only a single terminus of the fragments, and optionally wherein prior to digesting, the nucleic acids in the sample are each 35 bound at one terminus to a biotin molecule or to a hapten molecule, and said removing is carried out by a method which comprises contacting the nucleic acids in the sample with

streptavidin or avidin or with an anti-hapten antibody, respectively, affixed to a solid support.

This invention further provides in the first embodiment additional methods wherein said digesting with 5 said one or more restriction endonucleases leaves single-stranded nucleotide overhangs on the digested ends.

This invention further provides in the first embodiment additional methods wherein the step of probing further comprises hybridizing double-stranded adapter nucleic 10 acids with the digested sample fragments, each said adapter nucleic acid having an end complementary to said overhang generated by a particular one of the one or more restriction endonucleases, and ligating with a ligase a strand of said adapter nucleic acids to the 5' end of a strand of the 15 digested sample fragments to form ligated nucleic acid fragments.

This invention further provides in the first embodiment additional methods wherein said digesting with said one or more restriction endonucleases and said ligating 20 are carried out in the same reaction medium, and optionally wherein said digesting and said ligating comprises incubating said reaction medium at a first temperature and then at a second temperature, in which said one or more restriction endonucleases are more active at the first temperature than 25 the second temperature and said ligase is more active at the second temperature than the first temperature, or wherein said incubating at said first temperature and said incubating at said second temperature are performed repetitively.

This invention further provides in the first 30 embodiment additional methods wherein the step of probing further comprises prior to said digesting removing terminal phosphates from DNA in said sample by incubation with an alkaline phosphatase, and optionally wherein said alkaline phosphatase is heat labile and is heat inactivated prior to 35 said digesting.

This invention further provides in the first embodiment additional methods wherein said generating step

comprises amplifying the ligated nucleic acid fragments, and optionally wherein said amplifying is carried out by use of a nucleic acid polymerase and primer nucleic acid strands, said primer nucleic acid strands being capable of priming nucleic acid synthesis by said polymerase, and optionally wherein the primer nucleic acid strands have a G+C content of between 40% and 60%.

This invention further provides in the first embodiment additional methods wherein each said adapter 10 nucleic acid has a shorter strand and a longer strand, the longer strand being ligated to the digested sample fragments, and said generating step comprises prior to said amplifying step the melting of the shorter strand from the ligated fragments, contacting the ligated fragments with a DNA 15 polymerase, extending the ligated fragments by synthesis with the DNA polymerase to produce blunt-ended double stranded DNA fragments, and wherein the primer nucleic acid strands comprise a hybridizable portion the sequence of said longer strands, or optionally comprise the sequence of said longer 20 strands, each different primer nucleic acid strand priming amplification only of blunt ended double stranded DNA fragments that are produced after digestion by a particular restriction endonuclease.

This invention further provides in the first 25 embodiment additional methods wherein each primer nucleic acid strand is specific for a particular restriction endonuclease, and further comprises at the 3' end of and contiguous with the longer strand sequence the portion of the restriction endonuclease recognition site remaining on a 30 nucleic acid fragment terminus after digestion by the restriction endonuclease, or optionally wherein each said primer specific for a particular restriction endonuclease further comprises at its 3' end one or more nucleotides 3' to and contiguous with the remaining portion of the restriction 35 endonuclease recognition site, whereby the ligated nucleic acid fragment amplified is that comprising said remaining portion of said restriction endonuclease recognition site

contiguous to said one or more additional nucleotides, and optionally such that said primers comprising a particular said one or more additional nucleotides can be distinguishably detected from said primers comprising a 5 different said one or more additional nucleotides.

This invention further provides in the first embodiment additional methods wherein during said amplifying step the primer nucleic acid strands are annealed to the ligated nucleic acid fragments at a temperature that is less 10 than the melting temperature of the primer nucleic acid strands from strands complementary to the primer nucleic acid strands but greater than the melting temperature of the shorter adapter strands from the blunt-ended fragments.

This invention further provides in the first 15 embodiment additional methods wherein the recognition means are oligomers of nucleotides, nucleotide-mimics, or a combination of nucleotides and nucleotide-mimics, which are specifically hybridizable with the target subsequences, and optionally further provides additional methods wherein the 20 step of generating comprises amplifying with a nucleic acid polymerase and with primers comprising said oligomers, whereby fragments of nucleic acids in the sample between hybridized oligomers are amplified.

This invention further provides in the first 25 embodiment additional methods wherein said signals further comprise a representation of whether an additional target subsequence is present on said nucleic acid in the sample between said occurrences of target subsequences, and optionally wherein said additional target subsequence is 30 recognized by a method comprising contacting nucleic acids in the sample with oligomers of nucleotides, nucleotide-mimics, or mixed nucleotides and nucleotide-mimics, which are hybridizable with said additional target subsequence.

This invention further provides in the first 35 embodiment additional methods wherein the step of generating comprises suppressing said signals when an additional target subsequence is present on said nucleic acid in the sample

between said occurrences of target subsequences, and optionally wherein, when the step of generating comprises amplifying nucleic acids in the sample, said additional target subsequence is recognized by a method comprising 5 contacting nucleic acids in the sample with (a) oligomers of nucleotides, nucleotide-mimics, or mixed nucleotides and nucleotide-mimics, which hybridize with said additional target subsequence and disrupt the amplifying step; or (b) restriction endonucleases which have said additional target 10 subsequence as a recognition site and digest the nucleic acids in the sample at the recognition site.

This invention further provides in the first embodiment additional methods wherein the step of generating further comprises separating nucleic acid fragments by 15 length, and optionally wherein the step of generating further comprises detecting said separated nucleic acid fragments, and optionally wherein said detecting is carried out by a method comprising staining said fragments with silver, labeling said fragments with a DNA intercalating dye, or 20 detecting light emission from a fluorochrome label on said fragments.

This invention further provides in the first embodiment additional methods wherein said representation of the length between occurrences of target subsequences is the 25 length of fragments determined by said separating and detecting steps.

This invention further provides in the first embodiment additional methods wherein said separating is carried out by use of liquid chromatography, mass 30 spectrometry, or electrophoresis, and optionally wherein said electrophoresis is carried out in a slab gel or capillary configuration using a denaturing or non-denaturing medium.

This invention further provides in the first embodiment additional methods wherein a predetermined one or 35 more nucleotide sequences in said database are of interest, and wherein the target subsequences are such that said sequences of interest generate at least one signal that is

not generated by any other sequence likely to be present in the sample, and optionally wherein the nucleotide sequences of interest are a majority of sequences in said database.

This invention further provides in the first 5 embodiment additional methods wherein the target subsequences have a probability of occurrence in the nucleotide sequences in said database of from approximately 0.01 to approximately 0.30.

This invention further provides in the first 10 embodiment additional methods wherein the target subsequences are such that the majority of sequences in said database contain on average a sufficient number of occurrences of target subsequences in order to on average generate a signal that is not generated by any other nucleotide sequence in 15 said database, and optionally wherein the number of pairs of target subsequences present on average in the majority of sequences in said database is no less than 3, and wherein the average number of signals generated from the sequences in said database is such that the average difference between 20 lengths represented by the generated signals is greater than or equal to 1 base pair.

This invention further provides in the first embodiment additional methods wherein the target subsequences have a probability of occurrence, p , approximately given by 25 the solution of

$$\frac{R(R + 1)p^2}{2} = A$$

and

30

$$\frac{L}{Np^2} = B$$

wherein N = the number of different nucleotide sequences in 35 said database; L = the average length of said different nucleotide sequences in said database; R = the number of recognition means; A = the number of pairs of target

subsequences present on average in said different nucleotide sequences in said database; and B = the average difference between lengths represented by the signals generated from the nucleic acids in the sample, and optionally wherein A is 5 greater than or equal to 3 and wherein B is greater than or equal to 1.

This invention further provides in the first embodiment additional methods wherein the target subsequences are selected according to the further steps comprising 10 determining a pattern of signals that can be generated and the sequences capable of generating each such signal by simulating the steps of probing and generating applied to each sequences in said database of nucleotide sequences; ascertaining the value of said determined pattern according 15 to an information measure; and choosing the target subsequences in order to generate a new pattern that optimizes the information measure, and optionally wherein said choosing step selects target subsequences which comprise the recognition sites of the one or more restriction 20 endonucleases, and optionally wherein said choosing step selects target subsequences which comprise the recognition sites of the one or more restriction endonucleases contiguous with one or more additional nucleotides.

This invention further provides in the first 25 embodiment additional methods wherein a predetermined one or more of the nucleotide sequences present in said database of nucleotide sequences are of interest, and the information measure optimized is the number of such said sequences of interest which generate at least one signal that is not 30 generated by any other nucleotide sequence present in said database, and optionally wherein said nucleotide sequences of interest are a majority of the nucleotide sequences present in said database.

This invention further provides in the first 35 embodiment additional methods wherein said choosing step is by exhaustive search of all combinations of target subsequences of length less than approximately 10, or wherein

said step of choosing target subsequences is by a method comprising simulated annealing.

This invention further provides in the first embodiment additional methods wherein the step of searching 5 further comprises determining a pattern of signals that can be generated and the sequences capable of generating each such signal by simulating the steps of probing and generating applied to each sequence in said database of nucleotide sequences; and finding the one or more nucleotide sequences 10 in said database that are able to generate said one or more generated signals by finding in said pattern those signals that comprise a representation of the (i) the same lengths between occurrences of target subsequences as is represented by the generated signal and (ii) the same target subsequences 15 as is represented by the generated signal, or target subsequences that are members of the same sets of target subsequences represented by the generated signal.

This invention further provides in the first embodiment additional methods wherein the step of determining 20 further comprises searching for occurrences of said target subsequences or sets of target subsequences in nucleotide sequences in said database of nucleotide sequences; finding the lengths between occurrences of said target subsequences or sets of target subsequences in the nucleotide sequences of 25 said database; and forming the pattern of signals that can be generated from the sequences of said database in which the target subsequences were found to occur.

This invention further provides in the first embodiment additional methods wherein said restriction 30 endonucleases generate 5' overhangs at the terminus of digested fragments and wherein each double stranded adapter nucleic acid comprises a shorter nucleic acid strand consisting of a first and second contiguous portion, said first portion being a 5' end subsequence complementary to the 35 overhang produced by one of said restriction endonucleases; and a longer nucleic acid strand having a 3' end subsequence complementary to said second portion of the shorter strand.

This invention further provides in the first embodiment additional methods wherein said shorter strand has a melting temperature from a complementary strand of less than approximately 68°C, and has no terminal phosphate, and 5 optionally wherein said shorter strand is approximately 12 nucleotides long.

This invention further provides in the first embodiment additional methods wherein said longer strand has a melting temperature from a complementary strand of greater 10 than approximately 68°C, is not complementary to any nucleotide sequence in said database, and has no terminal phosphate, and optionally wherein said ligated nucleic acid fragments do not contain a recognition site for any of said restriction endonucleases, and optionally wherein said longer 15 strand is approximately 24 nucleotides long and has a G+C content between 40% and 60%.

This invention further provides in the first embodiment additional methods wherein said one or more restriction endonucleases are heat inactivated before said 20 ligating.

This invention further provides in the first embodiment additional methods wherein said restriction endonucleases generate 3' overhangs at the terminus of the digested fragments and wherein each double stranded adapter 25 nucleic acid comprises a longer nucleic acid strand consisting of a first and second contiguous portion, said first portion being a 3' end subsequence complementary to the overhang produced by one of said restriction endonucleases; and a shorter nucleic acid strand complementary to the 3' end 30 of said second portion of the longer nucleic acid stand.

This invention further provides in the first embodiment additional methods wherein said shorter strand has a melting temperature from said longer strand of less than approximately 68°C, and has no terminal phosphates, and 35 optionally wherein said shorter strand is 12 base pairs long.

This invention further provides in the first embodiment additional methods wherein said longer strand has

a melting temperature from a complementary strand of greater than approximately 68°C, is not complementary to any nucleotide sequence in said database, has no terminal phosphate, and wherein said ligated nucleic acid fragments do 5 not contain a recognition site for any of said restriction endonucleases, and optionally wherein said longer strand is 24 base pairs long and has a G+C content between 40% and 60%.

In a second embodiment, the invention provides a method for identifying or classifying a nucleic acid 10 comprising probing said nucleic acid with a plurality of recognition means, each recognition means recognizing a target nucleotide subsequence or a set of target nucleotide subsequences, in order to generate a set of signals, each signal representing whether said target subsequence or one of 15 said set of target subsequences is present or absent in said nucleic acid; and searching a nucleotide sequence database, said database comprising a plurality of known nucleotide sequences of nucleic acids that may be present in the sample, for sequences matching said generated set of signals, a 20 sequence from said database matching a set of signals when the sequence from said database (i) comprises the same target subsequences as are represented as present, or comprises target subsequences that are members of the sets of target subsequences represented as present by the generated sets of 25 signals and (ii) does not comprise the target subsequences represented as absent or that are members of the sets of target subsequences represented as absent by the generated sets of signals, whereby the nucleic acid is identified or classified, and optionally wherein the set of signals are 30 represented by a hash code which is a binary number.

This invention further provides in the second embodiment additional methods wherein the step of probing generates quantitative signals of the numbers of occurrences of said target subsequences or of members of said set of 35 target subsequences in said nucleic acid, and optionally wherein a sequence matches said generated set of signals when the sequence from said database comprises the same target

subsequences with the same number of occurrences in said sequence as in the quantitative signals and does not comprise the target subsequences represented as absent or target subsequences within the sets of target subsequences 5 represented as absent.

This invention further provides in the second embodiment additional methods wherein said plurality of nucleic acids are DNA.

This invention further provides in the second 10 embodiment additional methods wherein the recognition means are detectably labeled oligomers of nucleotides, nucleotide-mimics, or combinations of nucleotides and nucleotide-mimics, and the step of probing comprises hybridizing said nucleic acid with said oligomers, and optionally wherein said 15 detectably labeled oligomers are detected by a method comprising detecting light emission from a fluorochrome label on said oligomers or arranging said labeled oligomers to cause light to scatter from a light pipe and detecting said scattering, and optionally wherein the recognition means are 20 oligomers of peptido-nucleic acids, and optionally wherein the recognition means are DNA oligomers, DNA oligomers comprising universal nucleotides, or sets of partially degenerate DNA oligomers.

This invention further provides in the second 25 embodiment additional methods wherein the step of searching further comprises determining a pattern of sets of signals of the presence or absence of said target subsequences or said sets of target subsequences that can be generated and the sequences capable of generating each set of signals in said 30 pattern by simulating the step of probing as applied to each sequence in said database of nucleotide sequences; and finding one or more nucleotide sequences that are capable of generating said generated set of signals by finding in said pattern those sets that match said generated set, where a set 35 of signals from said pattern matches a generated set of signals when the set from said pattern (i) represents as present the same target subsequences as are represented as

present or target subsequences that are members of the sets of target subsequences represented as present by the generated sets of signals and (ii) represents as absent the target subsequences represented as absent or that are members 5 of the sets of target subsequences represented as absent by the generated sets of signals.

This invention further provides in the second embodiment additional methods wherein the target subsequences are selected according to the further steps comprising 10 determining (i) a pattern of sets of signals representing the presence or absence of said target subsequences or of said sets of target subsequences that can be generated, and (ii) the sequences capable of generating each set of signals in said pattern by simulating the step of probing as applied to 15 each sequence in said database of nucleotide sequences; ascertaining the value of said pattern generated according to an information measure; and choosing the target subsequences in order to generate a new pattern that optimizes the information measure.

20 This invention further provides in the second embodiment additional methods wherein the information measure is the number of sets of signals in the pattern which are capable of being generated by one or more sequences in said database, or optionally wherein the information measure is 25 the number of sets of signals in the pattern which are capable of being generated by only one sequence in said database.

This invention further provides in the second embodiment additional methods wherein said choosing step is 30 by a method comprising exhaustive search of all combination of target subsequences of length less than approximately 10, or optionally wherein said choosing step is by a method comprising simulated annealing.

This invention further provides in the second 35 embodiment additional methods wherein the step of determining by simulating further comprises searching for the presence or absence of said target subsequences or sets of target

subsequences in each nucleotide sequence in said database of nucleotide sequences; and forming the pattern of sets of signals that can be generated from said sequences in said database, and optionally where the step of searching is 5 carried out by a string search, and optionally wherein the step of searching comprises counting the number of occurrences of said target subsequences in each nucleotide sequence.

This invention further provides in the second 10 embodiment additional methods wherein the target subsequences have a probability of occurrence in a nucleotide sequence in said database of nucleotide sequences of from 0.01 to 0.6, or optionally wherein the target subsequences are such that the presence of one target subsequence in a nucleotide sequence 15 in said database of nucleotide sequences is substantially independent of the presence of any other target subsequence in the nucleotide sequence, or optionally wherein fewer than approximately 50 target subsequences are selected.

In a third embodiment, the invention provides a 20 programmable apparatus for analyzing signals comprising an inputting device for inputting one or more actual signals generated by probing a sample comprising a plurality of nucleic acids with recognition means, each recognition means recognizing a target nucleotide subsequence or a set of 25 target nucleotide subsequences, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a nucleic acid of said sample, and (ii) the identities of said target subsequences in said nucleic acid, or the identities of said sets of target 30 subsequences among which is included the target subsequences in said nucleic acid; a searching device operatively coupled to said accepting device for searching a sequence in a nucleotide sequence database for occurrences of said target subsequences or target subsequences that are members of said 35 sets of target subsequences, and for the length between such occurrences, said database comprising a plurality of known nucleotide sequences that may be present in said sample; a

comparing device operatively coupled to said accepting device and to said searching device for finding a match between said one or more actual signals and a sequence in said database, said one or more actual signals matching a sequence from said 5 database when the sequence from said database has both (i) the same length between occurrences of target subsequences as is represented by said one or more actual signals and (ii) the same target subsequences as is represented by said one or more actual signals or target subsequences that are members 10 of the same sets of target subsequences represented by said one or more actual signals; and a control device operatively coupled to said comparing device for causing said comparing to be done for sequences in the database and for outputting those database sequences that match said one or more actual 15 signals, and optionally wherein said searching device searches for said target subsequences or a set of target nucleotide subsequences in said database sequences by performing a string comparison of the nucleotides in said subsequences with those in said database sequence.

20 This invention further provides in the third embodiment that said control device further comprises causing said searching device to search substantially all sequences in said database in order to determine a pattern of signals that can be generated by probing said sample with said 25 recognition means, and wherein said control device further causes said comparing device to find any matches between said one or more actual signals and said pattern of signals, said one or more actual signals matching a signal in said pattern of signals when the signal from said pattern represents (i) 30 the same length between occurrences of target subsequences as is represented by said one or more actual signals and (ii) the same target subsequences as is represented by said one or more actual signals or target subsequences that are members of the same sets of target subsequences represented by said 35 one or more actual signals.

This invention further provides in the third embodiment that said sample of nucleic acids comprises cDNA

from RNA of a cell or tissue type, and said database comprises DNA sequences that are likely to be expressed by a cell or tissue type.

This invention further provides in the third embodiment a computer readable memory that can be used to direct a programmable apparatus to function for analyzing signals according to steps comprising inputting one or more actual signals generated by probing a sample comprising a plurality of nucleic acids with recognition means, each 10 recognition means recognizing a target nucleotide subsequence or a set of target nucleotide subsequences, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a nucleic acid of said sample, and (ii) the identities of said target 15 subsequences in said nucleic acid, or the identities of said sets of target subsequences among which is included the target subsequences in said nucleic acid; searching a sequence in a nucleotide sequence database for occurrences of said target subsequences or target subsequences that are 20 members of said sets of target subsequences, and for the length between such occurrences, said database comprising a plurality of known nucleotide sequences that may be present in said sample; matching said one or more actual signals and a sequence in said database when the sequence in said 25 database has both (i) the same length between occurrences of target subsequences as is represented by said one or more actual signals and (ii) the same target subsequences as is represented by said one or more actual signals, or target subsequences that are members of the same sets of target 30 subsequences as is represented by said one or more actual signals; and repetitively performing said searching and matching steps for the majority of sequences in the database and outputting those database sequences that match said one or more actual signals, or alternatively a computer readable 35 memory for directing a programmable apparatus to function in the manner of the third object.

In a fourth embodiment, the invention provides a programmable apparatus for selecting target subsequences comprising an initial selection device for selecting initial target subsequences or initial sets of target subsequences; a 5 first control device; a search device operatively coupled to said initial selection device and to said first control device (i) for searching sequences in a nucleotide sequence database for occurrences of said initial target subsequences or occurrences of target subsequences that are members of 10 said initial sets of target subsequences and for the length between such occurrences and (ii) for determining an initial pattern of signals that can be generated from said selected initial target subsequences or said initial sets of target subsequences, said database comprising a plurality of known 15 nucleotide sequences, said signals comprising a representation of (i) the length between said occurrences in a sequence in said database, and (ii) the identities of said initial target subsequences that occur in said sequence in said database, or the identities of target subsequences that 20 are members of the same initial sets of target subsequences that occur in said sequence in said database; and an ascertaining device operatively coupled to said searching device and to said first control device for ascertaining the value of said determined initial pattern according to an 25 information measure; and wherein said first control device causes further target subsequences to be selected and causes the search device to determine a further pattern of signals and the ascertaining device to ascertain a further value of said information measure and accepts the further target 30 subsequences when said further pattern optimizes said further value of said information measure.

This invention further provides in the fourth object that a predetermined one or more of the sequences in said database are of interest, and wherein said ascertaining 35 device ascertains the value of an information measure by counting the number of such sequences of interest which generate in said determined pattern at least one signal that

is not generated by any other sequence in said database, and optionally that said one or more of the sequences of interest comprise substantially all the sequences in said database.

This invention further provides in the fourth 5 embodiment that said first control device optimizes the value of said information measure according to a method of exhaustive search, wherein said first control device selects further target subsequences of length less than approximately 10 and accepts the further target subsequences if said 10 further value of said information measure is greater than the previous value.

This invention further provides in the fourth embodiment that said first control device optimizes the value of said information measure according to a method comprising 15 simulated annealing, wherein said first control device repeatedly selects further target subsequences and accepts the further target subsequences if said further value of said information measure is not decreased by greater than a probabilistic factor dependent on a simulated-temperature, 20 and wherein said programmable apparatus further comprises a second control device operatively coupled to said first control device for decreasing said simulated-temperature as said first control device selects further target subsequences, and optionally wherein said probabilistic 25 factor is an exponential function of the negative of the decrease in the information measure divided by said simulated-temperature.

This invention further provides in the fourth embodiment that the database comprises a majority of known 30 DNA sequences that are likely to be expressed by one or more cell types.

This invention further provides in the fourth embodiment a computer readable memory that can be used to direct a programmable apparatus to function for selecting 35 target subsequences according to steps comprising selecting initial target subsequences or initial sets of target subsequences; searching a sequence in a nucleotide sequence

database for occurrences of said initial target subsequences or occurrences of target subsequences that are members of said initial sets of target subsequences and for the length between such occurrences, said database comprising a 5 plurality of known nucleotide sequences that may be present in said sample; determining an initial pattern of signals that can be generated from said selected initial target subsequences or said initial sets of target subsequences, said signals comprising a representation of (i) the length 10 between said occurrences in a sequence in said database, and (ii) the identities of said initial target subsequences that occur in said sequence in said database, or the identities of target subsequences that are members of the initial sets of target subsequences that occur in said sequence in said 15 database; ascertaining the value of said determined initial pattern according to an information measure; and repetitively performing said selecting, searching, determining, and ascertaining steps to determine a further pattern of signals and a further value of said information measure, and 20 accepting the further target subsequences when said further pattern optimizes said further value of said information measure, or alternatively a computer readable memory for directing a programmable apparatus to function in the manner of the fourth object.

25 In a fifth embodiment, the invention provides a programmable apparatus for displaying data comprising a selecting device for selecting target subsequences or sets of target subsequences, such that recognition means for recognizing said target subsequences or said sets of target 30 subsequences can be used to generate signals by probing a sample comprising a plurality of nucleic acids, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a nucleic acid of said sample and (ii) the identities of said target 35 subsequences in said nucleic acid or the identities of said sets of target subsequences among which are included the target subsequences in said nucleic acid; an inputting device

for inputting one or more actual signals generated by probing said sample with said recognition means; an analyzing device for analyzing signals operatively coupled to said selecting and inputting devices that determines which sequences in a 5 nucleotide sequence database can generate said actual signals when subject to said recognition means, said database comprising a plurality of known nucleotide sequences that may be present in said sample; an input/output device operatively coupled to said selecting, inputting, and analyzing devices 10 that inputs user requests and controls the selecting device to select target subsequences or sets of target subsequences, controls the inputting device to accept actual signals, controls the analyzing device to find the sequences in said database that can generate said actual signals, and displays 15 output comprising said actual signals and said sequences in said database that can generate said actual signals.

This invention further provides in the fifth embodiment that said sample is a cDNA sample prepared from a tissue specimen, and the apparatus further comprises a 20 storage device operatively coupled to the input/output device for storing indications of the origin of said tissue specimen and information concerning said tissue specimen, and wherein said indications can be displayed upon user input, and optionally that the indications and information concerning 25 said tissue specimen comprises histological information comprising tissue images.

This invention further provides in the fifth embodiment additional apparatus further comprising one or more instrument devices for probing said sample with said 30 recognition means and for generating said actual signals; and a control device operatively coupled to said one or more instrument devices and to said input/output device for controlling the operation of said instrument devices, wherein said user can input control commands for control of said 35 instrument devices and receive output concerning the status of said instrument devices, and optionally wherein one or more of said selecting, inputting, analyzing, and

input/output devices are physically collocated with each other, or are physically spaced apart from each other and are connected by a communication medium for exchanges of commands and information.

5 This invention further provides in the fifth embodiment a computer readable memory that can be used to direct a programmable apparatus to function for displaying data according to steps comprising selecting target subsequences or sets of target subsequences, such that
10 recognition means for recognizing said target subsequences or said sets of target subsequences can be used to generate signals by probing a sample comprising a plurality of nucleic acids, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a
15 nucleic acid of said sample and (ii) the identities of said target subsequences in said nucleic acid or the identities of said sets of target subsequences among which are included the target subsequences in said nucleic acid inputting one or more actual signals generated by probing said sample with
20 said recognition means analyzing said one or more actual signals to determine which sequences in a nucleotide sequence database can generate said actual signals when subject to said recognition means, said database comprising a plurality of known nucleotide sequences that may be present in said
25 sample; and inputting user requests to control said selecting step to select target subsequences or sets of target subsequences, said inputting step to input actual signals, and said analyzing step to find the sequences in said database that can generate said actual signals, and
30 outputting in response to further user requests information comprising said actual signals and said sequences in said database that can generate said actual signals, or alternatively a computer readable memory for directing a programmable apparatus to function in the manner of the fifth
35 object.

In a sixth embodiment, the invention provides a method for identifying, classifying, or quantifying DNA

molecules in a sample of DNA molecules having a plurality of different nucleotide sequences, the method comprising the steps of digesting said sample with one or more restriction endonucleases, each said restriction endonuclease recognizing a subsequence recognition site and digesting DNA at said recognition site to produce fragments with 5' overhangs; contacting said fragments with shorter and longer oligodeoxynucleotides, each said shorter oligodeoxynucleotide hybridizable with a said 5' overhang and having no terminal phosphates, each said longer oligodeoxynucleotide hybridizable with a said shorter oligodeoxynucleotide; ligating said longer oligodeoxynucleotides to said 5' overhangs on said DNA fragments to produce ligated DNA fragments; extending said ligated DNA fragments by synthesis with a DNA polymerase to produce blunt-ended double stranded DNA fragments; amplifying said blunt-ended double stranded DNA fragments by a method comprising contacting said DNA fragments with a DNA polymerase and primer oligodeoxynucleotides, each said primer oligodeoxynucleotide having a sequence comprising that of one of the longer oligodeoxynucleotides; determining the length of the amplified DNA fragments; and searching a DNA sequence database, said database comprising a plurality of known DNA sequences that may be present in the sample, for sequences matching one or more of said fragments of determined length, a sequence from said database matching a fragment of determined length when the sequence from said database comprises recognition sites of said one or more restriction endonucleases spaced apart by the determined length, whereby DNA molecules in said sample are identified, classified, or quantified.

This invention further provides in the sixth embodiment additional methods wherein the sequence of each primer oligodeoxynucleotide further comprises 3' to and contiguous with the sequence of the longer oligodeoxynucleotide the portion of the recognition site of said one or more restriction endonucleases remaining on a DNA

fragment terminus after digestion, said remaining portion being 5' to and contiguous with one or more additional nucleotides, and wherein a sequence from said database matches a fragment of determined length when the sequence 5 from said database comprises subsequences that are the recognition sites of said one or more restriction endonucleases contiguous with said one or more additional nucleotides and when the subsequences are spaced apart by the determined length.

10 This invention further provides in the sixth embodiment additional methods wherein said determining step further comprises detecting the amplified DNA fragments by a method comprising staining said fragments with silver.

This invention further provides in the sixth 15 embodiment additional methods wherein said oligodeoxynucleotide primers are detectably labeled, wherein the determining step further comprises detection of said detectable labels, and wherein a sequence from said database matches a fragment of determined length when the sequence 20 from said database comprises recognition sites of the one or more restriction endonucleases, said recognition sites being identified by the detectable labels of said oligodeoxynucleotide primers, said recognition sites being spaced apart by the determined length, and optionally wherein 25 said determining step further comprises detecting the amplified DNA fragments by a method comprising labeling said fragments with a DNA intercalating dye or detecting light emission from a fluorochrome label on said fragments.

This invention further provides in the sixth 30 embodiment additional steps further comprising, prior to said determining step, the step of hybridizing the amplified DNA fragments with a detectably labeled oligodeoxynucleotide complementary to a subsequence, said subsequence differing from said recognition sites of said one or more restriction 35 endonucleases, wherein the determining step further comprises detecting said detectable label of said oligodeoxynucleotide, and wherein a sequence from said database matches a fragment

of determined length when the sequence from said database further comprises said subsequence between the recognition sites of said one or more restriction endonucleases.

This invention further provides in the sixth embodiment additional methods wherein the one or more restriction endonucleases are pairs of restriction endonucleases, the pairs being selected from the group consisting of Acc56I and HindIII, Acc65I and NgoMI, BamHI and EcoRI, BglII and HindIII, BglII and NgoMI, BsiWI and BspHI, BspHI and BstYI, BspHI and NgoMI, BsrGI and EcoRI, EagI and EcoRI, EagI and HindIII, EagI and NcoI, HindIII and NgoMI, NgoMI and NheI, NgoMI and SpeI, BglII and BspHI, Bsp120I and NcoI, BssHII and NgoMI, EcoRI and HindIII, and NgoMI and XbaI, or wherein the step of ligating is performed with T4 DNA ligase.

This invention further provides in the sixth embodiment additional methods wherein the steps of digesting, contacting, and ligating are performed simultaneously in the same reaction vessel, or optionally wherein the steps of digesting, contacting, ligating, extending, and amplifying are performed in the same reaction vessel.

This invention further provides in the sixth embodiment additional methods wherein the step of determining the length is performed by electrophoresis.

This invention further provides in the sixth embodiment additional methods wherein the step of searching said DNA database further comprises determining a pattern of fragments that can be generated and for each fragment in said pattern those sequences in said DNA database that are capable of generating the fragment by simulating the steps of digesting with said one or more restriction endonucleases, contacting, ligating, extending, amplifying, and determining applied to each sequence in said DNA database; and finding the sequences that are capable of generating said one or more fragments of determined length by finding in said pattern one or more fragments that have the same length and recognition sites as said one or more fragments of determined length.

This invention further provides in the sixth embodiment additional methods wherein the steps of digesting and ligating go substantially to completion.

This invention further provides in the sixth 5 embodiment additional methods wherein the DNA sample is cDNA prepared from mRNA, and optionally wherein the DNA is of RNA from a tissue or a cell type derived from a plant, a single celled animal, a multicellular animal, a bacterium, a virus, a fungus, a yeast, or a mammal, and optionally wherein the 10 mammal is a human, and optionally wherein the mammal is a human having or suspected of having a diseased condition, and optionally wherein the diseased condition is a malignancy.

In a seventh embodiment, this invention provides additional methods for identifying, classifying, or 15 quantifying DNA molecules in a sample of DNA molecules with a plurality of nucleotide sequences, the method comprising the steps of digesting said sample with one or more restriction endonucleases, each said restriction endonuclease recognizing a subsequence recognition site and digesting DNA to produce 20 fragments with 3' overhangs; contacting said fragments with shorter and longer oligodeoxynucleotides, each said longer oligodeoxynucleotide consisting of a first and second contiguous portion, said first portion being a 3' end subsequence complementary to the overhang produced by one of 25 said restriction endonucleases, each said shorter oligodeoxynucleotide complementary to the 3' end of said second portion of said longer oligodeoxynucleotide stand; ligating said longer oligodeoxynucleotide to said DNA fragments to produce a ligated fragment; extending said 30 ligated DNA fragments by synthesis with a DNA polymerase to form blunt-ended double stranded DNA fragments; amplifying said double stranded DNA fragments by use of a DNA polymerase and primer oligodeoxynucleotides to produce amplified DNA fragments, each said primer oligodeoxynucleotide having a 35 sequence comprising that of a longer oligodeoxynucleotides; determining the length of the amplified DNA fragments; and searching a DNA sequence database, said database comprising a

plurality of known DNA sequences that may be present in the sample, for sequences matching one or more of said fragments of determined length, a sequence from said database matching a fragment of determined length when the sequence from said database comprises recognition sites of said one or more restriction endonucleases spaced apart by the determined length, whereby DNA sequences in said sample are identified, classified, or quantified.

In an eighth embodiment, this invention provides additional methods of detecting one or more differentially expressed genes in an *in vitro* cell exposed to an exogenous factor relative to an *in vitro* cell not exposed to said exogenous factor comprising performing the methods of the first embodiment of this invention wherein said plurality of nucleic acids comprises cDNA of RNA of said *in vitro* cell exposed to said exogenous factor; performing the methods of the first embodiment of this invention wherein said plurality of nucleic acids comprises cDNA of RNA of said *in vitro* cell not exposed to said exogenous factor; and comparing the identified, classified, or quantified cDNA of said *in vitro* cell exposed to said exogenous factor with the identified, classified, or quantified cDNA of said *in vitro* cell not exposed to said exogenous factor, whereby differentially expressed genes are identified, classified, or quantified.

In a ninth embodiment, this invention provides additional methods of detecting one or more differentially expressed genes in a diseased tissue relative to a tissue not having said disease comprising performing the methods of the first embodiment of this invention wherein said plurality of nucleic acids comprises cDNA of RNA of said diseased tissue such that one or more cDNA molecules are identified, classified, and/or quantified; performing the methods of the first embodiment of this invention wherein said plurality of nucleic acids comprises cDNA of RNA of said tissue not having said disease such that one or more cDNA molecules are identified, classified, and/or quantified; and comparing said identified, classified, and/or quantified cDNA molecules of

said diseased tissue with said identified, classified, and/or quantified cDNA molecules of said tissue not having the disease, whereby differentially expressed cDNA molecules are detected.

5 This invention further provides in the ninth embodiment additional methods wherein the step of comparing further comprises finding cDNA molecules which are reproducibly expressed in said diseased tissue or in said tissue not having the disease and further finding which of 10 said reproducibly expressed cDNA molecules have significant differences in expression between the tissue having said disease and the tissue not having said disease, and optionally wherein said finding cDNA molecules which are reproducibly expressed and said significant differences in 15 expression of said cDNA molecules in said diseased tissue and in said tissue not having the disease are determined by a method comprising applying statistical measures, and optionally wherein said statistical measures comprise determining reproducible expression if the standard deviation 20 of the level of quantified expression of a cDNA molecule in said diseased tissue or said tissue not having the disease is less than the average level of quantified expression of said cDNA molecule in said diseased tissue or said tissue not having the disease, respectively, and wherein a cDNA molecule 25 has significant differences in expression if the sum of the standard deviation of the level of quantified expression of said cDNA molecule in said diseased tissue plus the standard deviation of the level of quantified expression of said cDNA molecule in said tissue not having the disease is less than 30 the absolute value of the difference of the level of quantified expression of said cDNA molecule in said diseased tissue minus the level of quantified expression of said cDNA molecule in said tissue not having the disease.

 This invention further provides in the ninth 35 embodiment additional methods wherein the diseased tissue and the tissue not having the disease are from one or more mammals, and optionally wherein the disease is a malignancy,

and optionally wherein the disease is a malignancy selected from the group consisting of prostrate cancer, breast cancer, colon cancer, lung cancer, skin cancer, lymphoma, and leukemia.

5 This invention further provides in the ninth embodiment additional methods wherein the disease is a malignancy and the tissue not having the disease has a premalignant character.

In a tenth embodiment, this invention provides 10 methods of staging or grading a disease in a human individual comprising performing the methods of the first embodiment of this invention in which said plurality of nucleic acids comprises cDNA of RNA prepared from a tissue from said human individual, said tissue having or suspected of having said 15 disease, whereby one or more said cDNA molecules are identified, classified, and/or quantified; and comparing said one or more identified, classified, and/or quantified cDNA molecules in said tissue to the one or more identified, classified, and/or quantified cDNA molecules expected at a 20 particular stage or grade of said disease.

In an eleventh embodiment, this invention provides additional methods for predicting a human patient's response to therapy for a disease, comprising performing the methods of the first embodiment of this invention in which said 25 plurality of nucleic acids comprises cDNA of RNA prepared from a tissue from said human patient, said tissue having or suspected of having said disease, whereby one or more cDNA molecules in said sample are identified, classified, and/or quantified; and ascertaining if the one or more cDNA 30 molecules thereby identified, classified, and/or quantified correlates with a poor or a favorable response to one or more therapies, and optionally which further comprises selecting one or more therapies for said patient for which said identified, classified, and/or quantified cDNA molecules 35 correlates with a favorable response.

In a twelfth embodiment, this invention provides additional methods for evaluating the efficacy of a therapy

in a mammal having a disease, the method comprising performing the methods of the first embodiment of this invention wherein said plurality of nucleic acids comprises cDNA of RNA of said mammal prior to a therapy; performing the 5 method of the first embodiment of this invention wherein said plurality of nucleic acids comprises cDNA of RNA of said mammal subsequent to said therapy; comparing one or more identified, classified, and/or quantified cDNA molecules in said mammal prior to said therapy with one or more 10 identified, classified, and/or quantified cDNA molecules of said mammal subsequent to therapy; and determining whether the response to therapy is favorable or unfavorable according to whether any differences in the one or more identified, classified, and/or quantified cDNA molecules after therapy 15 are correlated with regression or progression, respectively, of the disease, and optionally wherein the mammal is a human.

In a thirteenth embodiment, this invention provides a kit comprising one or more containers having one or more restriction endonucleases; one or more containers having one 20 or more shorter oligodeoxynucleotide strands; one or more containers having one or more longer oligodeoxynucleotide strands hybridizable with said shorter strands, wherein either the longer or the shorter oligodeoxynucleotide strands each comprise a sequence complementary to an overhang 25 produced by at least one of said one or more restriction endonucleases; and instructions packaged in association with said one or more containers for use of said restriction endonucleases, shorter strands, and longer strands for identifying, classifying, or quantifying one or more DNA 30 molecules in a DNA sample, said instructions comprising (i) digest said sample with said restriction endonucleases into fragments, each fragment being terminated on each end by a recognition site of said one or more restriction endonucleases; (ii) contact said shorter and longer strands 35 and said digested fragments to form double stranded DNA adapters annealed to said digested fragments, (iii) ligate said longer strand to said fragments; (iv) generate one or

more signals by separating and detecting such of said fragments that are digested on each end, each signal comprising a representation of the length of the fragment and the identity of the recognition sites on both termini of the 5 fragments; and (v) search a nucleotide sequence database to determine sequences that match or the absence of any sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences of nucleic acids that may be present in the sample, a 10 sequence from said database matching a generated signal when the sequence from said database has both (i) the same length between occurrences of said recognition sites of said one or more restriction endonucleases as is represented by the generated signal and (ii) the same recognition sites of said 15 one of more restriction endonucleases as is represented by the generated signal.

This invention further provides in the thirteenth embodiment a kit wherein said one or more restriction endonucleases generate 5' overhangs at the terminus of 20 digested fragments, wherein each said shorter oligodeoxynucleotide strand consists of a first and second contiguous portion, said first portion being a 5' end subsequence complementary to the overhang produced by one of said restriction endonucleases, and wherein each said longer 25 oligodeoxynucleotide strand comprises a 3' end subsequence complementary to said second portion of said shorter oligodeoxynucleotide strand, or optionally wherein said one or more restriction endonucleases generate 3' overhangs at the terminus of the digested fragments, wherein each said 30 longer oligodeoxynucleotide strand consists of a first and second contiguous portion, said first portion being a 3' end subsequence complementary to the overhang produced by one of said restriction endonucleases, and wherein each said shorter oligodeoxynucleotide strand is complementary to the 3' end of 35 said second portion of said longer oligodeoxynucleotide stand.

This invention further provides in the thirteenth embodiment a kit wherein said instructions further comprise those signals expected from one or more DNA molecules of interest when said sample is digested with a particular one 5 or more restriction endonucleases selected from among said one or more restriction endonucleases in said kit, and optionally wherein said one or more DNA molecules of interest are cDNA molecules differentially expressed in a disease condition.

10 This invention further provides in the thirteenth embodiment a kit wherein the restriction endonucleases are selected from the group consisting of Acc65I, AflIII, AgeI, ApaLI, ApoI, AscI, AvrI, BamHI, BclI, BglII, BsiWI, Bsp120I, BspEI, BspHI, BsrGI, BssHII, BstYI, EaqI, EcoRI, HindIII, 15 MluI, NcoI, NgoMI, NheI, NotI, SpeI, and XbaI.

This invention further provides in the thirteenth embodiment a kit further comprising one or more containers having one or more double stranded adapter DNA molecules formed by annealing said longer and said shorter 20 oligonucleotide strands.

This invention further provides in the thirteenth embodiment a kit further comprising the computer readable memory of claim 106, or optionally further comprising the computer readable memory of claim 114, or optionally further 25 comprising the computer readable memory of claim 122.

This invention further provides in the thirteenth embodiment a kit further comprising in a container a DNA ligase, or optionally further comprising in a container a phosphatase capable of removing terminal phosphates from a 30 DNA sequence.

This invention further provides in the thirteenth embodiment a kit further comprising one or more primers, each said primer consisting of a single stranded oligodeoxynucleotide comprising the sequence of one of said 35 longer strands; and a DNA polymerase, and optionally wherein each of said one or more primers further comprises (a) a first subsequence that is the portion of the recognition site

of one of said one or more restriction endonucleases remaining at the terminus of a fragment after digestion, and (b) a second subsequence of one or two additional nucleotides contiguous with and 3' to said first subsequence, wherein 5 said primer is detectably labeled such that primers with differing said one or two additional nucleotides have different labels that can be distinguishably detected.

This invention further provides in the thirteenth embodiment a kit wherein said instructions further comprise: 10 detect such of said fragments digested on each end by a method comprising staining said fragments with silver, labeling said fragments with a DNA intercalating dye, or detecting light emission from a fluorochrome label on said fragments.

15 This invention further provides in the thirteenth embodiment a kit further comprising reagents for performing a cDNA sample preparation step; reagents for performing a step of digestion by one or more restriction endonucleases; reagents for performing a ligation step; and reagents for 20 performing a PCR amplification step.

4. BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood by 25 reference to the accompanying drawings, following description, and appended claims, where:

Fig. 1 illustrates exemplary results of the signals generated by QEA™ methods of this invention;

Figs. 2A, 2B, and 2C illustrate DNA adapters for an 30 RE/ligation implementation of QEA™ methods of this invention, where the restriction endonucleases generate 5' overhangs, open blocks indicating strands of DNA;

Figs. 3A and 3B illustrate the DNA adapters for an RE/ligation implementation of QEA™ methods of this invention, 35 where the restriction endonucleases generate 3' overhangs;

Figs. 4A, 4B, and 4C illustrate an exemplary biotin alternative embodiment of QEA™ methods;

Fig. 5 illustrates the DNA primers for a PCR embodiment of QEA™ methods;

Figs. 6A and 6B illustrate a method for DNA sequence database selection according to this invention;

5 Fig. 7 illustrates an exemplary experimental description for QEA™ embodiments of this invention;

Figs. 8A and 8B illustrate an overview of a method for determining a simulated database of experimental results for QEA™ embodiments of this invention;

10 Fig. 9 illustrates the detail of a method for simulating a QEA™ reaction;

Figs. 10A-F illustrate exemplary results of the action of the method of Fig. 9;

15 Fig. 11 illustrates the detail of a method for determining a simulated database of experimental results for a QEA™ embodiment of this invention;

Figs. 12A, 12B, and 12C illustrate an exemplary computer system apparatus, and an alternative embodiment, implementing methods of this invention;

20 Fig. 13A illustrates exemplary detail of an experimental design method for QEA™ and CC embodiments of this invention and Fig. 13B illustrates exemplary detail of an experimental design method for a QEA™ embodiment of this invention;

Fig. 14 illustrates an exemplary method for ordering the 25 DNA sequences found to be likely causes of a QEA™ signal in the order of their likely presence in the sample;

Fig. 15 illustrates the detail of a method for determining a simulated database of experimental results for a CC embodiment of this invention;

30 Figs. 16A, 16B, 16C, and 16D illustrate exemplary reaction temperature profiles for preferred manual and automated implementations of a preferred RE embodiment of a QEA™ method; and

Figs. 17A-F illustrate the SEQ-QEA™ alternative 35 embodiment of the RE/ligase embodiment of QEA™.

5. DETAILED DESCRIPTION

According to the present invention, to uniquely identify an expressed nucleotide or gene sequence, full or partial, as well as many components of genomic DNA, it is not necessary to determine the actual, complete nucleotide sequences. Full sequences provide far more information than is needed to merely classify or determine a sequence according to this invention. For example, in the human genome, it is known that there are approximately 10^5 expressed genes. Since the average length of a coding sequence is approximately 2000 nucleotides, the total number of possible sequences is approximately 4^{2000} , or about 10^{1200} . The actual number of expressed human genes is an unimaginably small fraction (10^{-1195}) of the total number of possible DNA sequences. Even sequencing a 50 bp fragment of a cDNA sequence generates about 10^{25} times more information than is needed for classification of that sequence. Use of the present invention allows direct determination of sequences in a sample with far less information than either a complete or a partial sequence determination of a sample by making use of a database of sequences likely to be present in the sample. If such a database is not available, sequences in the sample can nevertheless be separately classified.

More generally, the invention is adaptable to analyzing the sequences of any biopolymer, built of a small number of repeating units, whose naturally occurring representatives are far fewer than the number of possible, physical polymers and in which small subsequences can be recognized. Thus it is applicable to not only naturally occurring DNA polymers but also to naturally occurring RNA polymers, proteins, glycans, etc.

In computer science, codes which compactly identify a few members from among a large set of possibilities are called hash codes. An object of this invention is to construct hash codes for expressed DNA sequences, or alternatively for any other existing set of DNA sequences. In a fully populated hash code without any unassigned code

words, all human genes could be coded by an approximately 17 bit binary number ($2^{17} = 1.3 \times 10^5$). A 20 bit code would be about 10% filled or 90% sparse ($2^{20} = 1.0 \times 10^6$).

In this invention codes are constructed from one or 5 more signals which represent the presence of short nucleic acid (preferably DNA) subsequences (hereinafter called "target subsequences") in the sample sequence and, preferably, in a QEA™ embodiment, include a representation of the length along the sample sequence between adjacent target 10 subsequences. In some embodiments, the presence of target subsequences is directly recognized by direct subsequence recognition means, including, but not limited to, REs and other DNA binding proteins, which bind and/or react with target subsequences, and oligomers of, for example, PNAs or 15 DNAs, which hybridize to target subsequences. In other embodiments, the presence of effective target subsequences is recognized indirectly as a result of applying protocols, perhaps involving multiple DNA binding proteins together with hybridizing oligomers. In this latter case, each of the 20 multiple proteins or oligomers can recognize a separate subsequence and the effective target subsequence can be the combination of the separate subsequences. A preferable combination is subsequence concatenation in the situation where all the separately recognized subsequences are 25 adjacent. Such effective target subsequences can have advantageous properties not achievable by, for example, REs or PNA oligomers alone. However, this invention, and particularly its computer methods, are adaptable to any acceptable subsequence recognition means available in the 30 art. The computer implemented analysis and design methods treat target subsequences and effective target subsequences in the same manner. Such acceptable subsequence recognition means preferably precisely and reproducibly recognize target subsequences and generate a recognition signal with adequate 35 signal to noise ratio and further preferably provide information on the length between target subsequences.

The signals of this invention, which contain representations of target subsequence occurrences and, preferably, representations of the length between target subsequence occurrences, can differ in various embodiments of this invention. In some embodiments, target subsequences are exactly recognized, for example, where REs are the recognition means, and subsequence representation can be the unique identity of the subsequences. In other embodiments, target subsequence recognition is less exact, for example, where short oligomers are used, and this representation can be "fuzzy". In the case of short oligomer, a fuzzy representation can consist of all subsequences which differ by one nucleotide from a target subsequence, each such subsequence, perhaps weighted by the probability that each member of the set is the target subsequence. Further, length representation may depend on the separation and detection means used to generate the signals. In the case of electrophoretic separation, the length observed electrophoretically may need to be corrected, perhaps up to 5 to 10%, for mobility differences due to average base composition differences or due to effects of labeling moieties used for detection. As these corrections often are not be known until the total sample sequence is determined, the length representation of the signal can use the electrophoretic length in bp and not the physical length in bp. For simplicity and without limitation, in the following description unless otherwise noted the signals are presumed to represent physically correct lengths, as if generated by precise recognition means with a length determined by error or bias free separation and detection means. However, in particular embodiments, target subsequences can be represented in a fuzzy manner and length, if present, can include separation and detection bias.

Target subsequences recognized are typically contiguous. This is typical for REs adaptable to this invention. However, this invention is adaptable to means recognizing discontiguous target subsequences or

discontiguous effective target subsequences. For example, oligomers recognizing discontinuous subsequences can be constructed by inserting degenerate nucleotides in a discontinuous region. A set of 16 oligomers recognizing AGC-5-TAT, with a two nucleotide discontiguous region, can be constructed according to the schema TCGNNATA, where N is any nucleotide. Alternately, such discontiguous subsequences can be recognized by one oligomer of the form TCGiiATA, where "i" is inosine, or any other "universal" nucleotide, capable of 10 hybridizing with any naturally occurring base.

Typically and without limitation, however, the invention is applied to the analysis of cDNA samples synthesized from any *in vivo* or *in vitro* sources of RNA. cDNA can be synthesized either from total cellular RNA, from 15 poly(A)⁺ RNA, or from specific sub-pools of RNA. Such RNA sub-pools can be produced by RNA pre-purification, for example, separation of mRNA of the endoplasmic reticulum from cytoplasmic mRNA enriches mRNA primarily encoding for cell surface or extracellular proteins (Celis et al., 1994, *Cell Biology*, Academic Press, New York, NY). Such enriched mRNAs have increased diagnostic or therapeutic utility due, for example, to their encoded protein's cell-surface or extracellular roles, such as being a receptor. Such pre-purified RNA pools can be used in all embodiments of this 20 invention. First strand cDNA synthesis can be performed by any method known in the art and can use any priming method known in the art. For example, first strand synthesis primers can be oligo(dT) primers, random hexamer primers, phasing primers, mixtures thereof, etc. In particular, 25 phasing primers, containing either an A, C, or G at the 3' end, can be used in separate cDNA synthesis reactions to split the cDNA first strands into 3 pools, each generated from poly(A)⁺ mRNA having a T, G, or C, respectively, 5' to the poly(A)⁺ tail. Twelve pools can be synthesized by using 30 the 12 possible oligo(dT) phasing primers not containing a 3-terminal thymidine. Further, cDNA can be synthesized by 35

methods biased to producing full-length cDNAs, e.g. by requiring presence of the 5'-cap in the source cap mRNA.

Two specific embodiments of the invention are respectively termed "quantitative expression analysis"

5 ("QEATM") and "colony calling" ("CC"). The specific embodiment known as QEATM probes a sample with recognition means generating signals that preferably comprise an indication of the presence of a first target subsequence, an indication of the presence of a second target subsequence, 10 and a representation of the length between the target subsequences in the sample nucleic acid sequence. If the first strand of target subsequences occur more than once in a single nucleic acid in the sample, more than one signal is generated, each signal comprising the length between adjacent 15 occurrences of the target subsequences.

QEATM embodiments are preferred for classifying and determining sequences in mixtures of cDNAs, but is also adaptable to samples with only one cDNA. It affords the relative advantage over prior art methods that cloning of 20 sample nucleic acids is not required. Typically, enough pairs of target subsequences can be chosen so that sufficient distinguishable signals can be generated to determine one to all the sequences in the sample mixture. For example, first, any pair of target subsequences may occur more than once in a 25 single DNA molecule to be analyzed, thereby generating several signals with differing lengths from one DNA molecule. Second, even if a pair of target subsequences occurs only once in two different DNA molecules to be analyzed, the lengths between the hits may differ and thus distinguishable 30 signals may be generated.

The target subsequences used in QEATM are preferably optimally chosen by the computer implemented methods of this invention in view of DNA sequence databases containing sequences likely to occur in the sample to be analyzed. In 35 the case of human cDNA, efforts of the Human Genome Project in the United States, efforts abroad, and efforts of private companies in the sequencing of the human genome sequences,

both expressed and genetic, are being collected in several available databases (listed in Sec. 5.1).

Typically, QEA™ can be performed in a "query mode" or in a "tissue mode." A query mode experiment focuses on 5 determining the expression of a limited number of genes, perhaps 1 - 100, of interest and of known sequence. A minimal number of target subsequences are chosen to generate signals, with the goal that each of the limited number of genes is discriminated from all the other genes likely to 10 occur in the sample by at least one unique signal. In other words, such a QEA™ experiment is designed so that each gene of interest generates at least one signal unique to it (a "good" gene, see *infra*). A QEA™ tissue mode experiment focuses on determining the expression of as many as possible, 15 preferably a majority, of the genes expressed in a tissue or other sample, without the need for any prior knowledge or interest in their expression. Target subsequences are optimally chosen to discriminate the maximum number of sample DNA sequences into classes comprising one or preferably at 20 most a few sequences. Preferably, enough signals are produced and detected so that the computer methods of this invention can uniquely determine the expression of a majority, or more preferably most, of the genes expressed in a tissue. In both modes, signals are generated and detected 25 as determined by the threshold and sensitivity of a particular experiment. Some important determinants of threshold and sensitivity are the initial amount of mRNA and thus of cDNA, the amount of molecular amplification performed during the experiment, and the sensitivity of the detection 30 means.

QEA™ signals are generated by methods comprising a recognition means for target subsequences that include, but are not limited to one or more REs in a preferred RE/ligase embodiment or nucleotide oligomer primers in an alternative 35 PCR embodiment. In both embodiments, this invention contemplates embodiments which select certain classes of QEA™ reaction products and remove unwanted products. These

embodiments advantageously increase the signal to noise ("s/n") ratio of the resulting signals.

In general, the RE/ligase method proceeds according to the following steps. The method employs recognition 5 reactions with one, a pair, or more REs which recognize target subsequences with high specificity and cut the sequence at the recognition sites leaving fragments with sticky overhangs characteristic of the particular RE. To each sticky overhang, specially constructed, labeled 10 amplification primers are ligated with the aid of shorter linkers in a manner so that the particular RE making the cut, and thus the particular target subsequence, can be later identified. A DNA polymerase then forms blunt-ended DNA 15 fragments. These fragments are then PCR amplified using the same special labeled primers for a number of cycles preferably just sufficient to detect signals from all fragments of interest and just sufficient to make signals from fragments not of interest, e.g., the linearly amplifying singly cut fragments, relatively insignificant. The 20 amplified labeled fragments are then separated by length using gel electrophoresis in either denaturing or non-denaturing conditions and the length and labeling of the fragments is optically detected. Optionally, single stranded fragments can be removed by a binding hydroxyapatite, or 25 other single strand specific, column or by digestion by a single strand specific nuclease. Also, this invention is adaptable to other functionally equivalent amplification and length separation means. In this manner, the identity of the REs cutting a fragment, and thereby the subsequences present, 30 as well as the length between the cuts is determined.

The RE/ligase embodiment is adaptable to several embodiments which enhance quantitative characteristics of QEA™ signals or which increase sample sequence discrimination. Certain embodiments use a removal means to 35 improve such quantitative characteristics as sensitivity and linear responsiveness. One or more of the special, labeled amplification primers described above and used in the PCR

amplification step can have attached removal means comprising a capture moiety attached to the primer and a binding partner attached to a solid support, e.g., biotin and streptavidin beads. In this manner certain products of the PCR reactions, 5 e.g., fragments cut with different REs at each end, can be separated and purified from background fragments. Such purified fragments can thereby be detected with increased sensitivity. For example, fragments cut with pairs of different REs on both ends are preferably separated since 10 such fragments contain the majority of signals. With N REs, there are $(N-1)/2$ pairs with different REs but only N pairs with the same RE.

Alternatively, cDNA is synthesized from an mRNA sample with synthesis primers at least one of which is 15 biotinylated. In the case where only one synthesis primer is biotinylated, the cDNA is then cyclized. In any case, the cDNA is then cut with a one or a pair of REs, and the special, labeled amplification primers are ligated to the cut ends with the aid of shorter linkers as previously discussed. 20 The singly cut ends attached to the biotinylated cDNA synthesis primers are removed with streptavidin or avidin beads leaving highly pure double cut cDNA fragments with ligated amplification primers, but with minimal singly cut and labeled background fragments. With sufficiently 25 sensitive detection means, these pure doubly cut and labeled fragments can be directly detected, after separation by length (e.g., by electrophoresis or column chromatography), without amplification. If amplification is needed, absence of the DNA singly cut background fragments improves signal to 30 noise ratio resulting in fewer necessary amplification cycles. Thereby, PCR amplification bias is decreased or eliminated and linear responsiveness of QEA™ signals to input mRNA amounts is improved.

Other RE/ligase embodiments increase sample 35 sequence discrimination in QEA™ experiments, for example, by recognizing target subsequences longer or less limited than those recognized by REs, or by recognizing third subsequences

interior to cut fragments. This added information can often discriminate two sample sequences producing fragments having identical original end subsequences and lengths. It is used in the computer implemented database lookup methods of this invention in a manner similar to the use of target subsequences. In one embodiments, the target subsequences recognized can be effectively lengthened by using an amplification primer with an internal Type IIS RE recognition site so positioned that the Type IIS RE cuts the amplified fragments in a manner producing a second overhang contiguous with the recognition site of the initial RE. The sequence of the second overhang concatenated with the initial target end subsequence produces an effectively longer target subsequence. Alternatively, an effectively longer target subsequence can be recognized by using phasing primers during PCR amplification. The PCR amplification step can be divided into several pools with each pool using one phasing amplification primer constructed so as to recognize one or more additional nucleotides beyond the original RE recognition site. These additional nucleotides then contribute to an effectively longer target subsequence.

A third subsequence internal to a fragment can be recognized by a distinctively labeled probe binding or hybridizing with the third subsequence. Such a probe added before detection generates unique signals from the fragment containing that subsequence. Alternatively, a probe can suppress signals from fragments with the third subsequence. For example, a probe added before the PCR amplification step and which prevents amplification of a fragment with the third subsequence thereby removes and suppresses any signal from such fragments. Such a probe can be without limitation either an RE for recognizing and cutting the fragment with the third subsequence or a PNA or modified DNA oligomer, which cannot serve as a PCR primer, for hybridizing with the third subsequence. Also, a third subsequence can be the sequence of the overhang produced by a Type IIS RE cutting the amplification primers sufficiently close to their 3' ends

so that the resulting overhang is not contiguous with the recognition sequence of the initial RE.

Further, various embodiments for improving the quantitative characteristics of QEA™ experiments and for 5 improving the discrimination of sample sequences can be combined in advantageous fashions to achieve both improvements in the same experiment. For example, removal means to increase the s/n ratio is combined with a Type IIS RE cutting the amplification primers to increase sample 10 sequence discrimination in an embodiment called SEQ-QEA™.

In a preferred PCR method for QEA™, a suitable collection of target subsequences is chosen by the computer implemented QEA™ experimental design methods, and PCR primers distinctively labeled with fluorochromes are synthesized to 15 hybridize with these target subsequences. The primers are designed as described in Sec. 5.3 to reliably recognize short subsequences while achieving a high specificity in PCR amplification. Using these primers, a minimum number of PCR amplification steps amplifies those fragments between the 20 primed subsequences existing in DNA sequences in the sample, thereby recognizing the target subsequences. The labeled, amplified fragments are then separated by gel electrophoresis and detected. Further, the PCR embodiment is adaptable to the same embodiment previously discussed with respect to the 25 RE/ligase embodiment.

The signals generated from the recognition reactions of a QEA™ experiment are analyzed by computer methods of this invention. The analysis methods simulate a QEA™ experiment using a database either of substantially all 30 known DNA sequences or of substantially all, or at least a majority of, the DNA sequences likely to be present in a sample to be analyzed and a description of the reactions to be performed. The simulation results in a digest database which contains for each possible signal that can be generated 35 the database sequences responsible for that signal. Thereby, finding the sequences that can generate a signal involves a look-up in the simulated digest database. Computer

implemented design methods optimize the choice of target subsequences in QEA™ reactions in order to maximize the information produced in an experiment. For the tissue mode, the methods maximize the number of sequences having unique 5 signals by which their quantitative presence can be unambiguously determined. For the query mode, the methods maximize only the number of sequences of interest having unique signals, ignoring recognition of other sequences that might be present in a sample.

10 The second specific embodiment known as colony calling ("CC") generates subsequence occurrence data without length information. Since this method requires only hybridizations, it is preferred for gene identification in arrayed single-sequence clones constructed from a tissue 15 library. This embodiment constructs a binary code in which each bit of the code represents the presence or absence of one target subsequence. By probing four to eight target subsequences in parallel, such as by using distinguishable fluorescent labeling of the multiple probes, in view of the 20 adequacy of a 20 bit code, the presence or absence of any expressed human gene should be determinable in just three to five separate probe steps. Such a compact method with such economy in signal generation is highly useful. Alternatively, recent real time hybridization detection 25 methods (Stimson et al., 1995, Proc. Natl. Acad. Sci. USA, 92:6379-6383) based on optical wave guides can be used for detection. These methods make hybridization detection more efficient both by eliminating the washing step otherwise needed between hybridization and detection and by speeding up 30 the detection step.

The hash code generated by the probe hybridization reactions is interpreted by computer implemented methods of this invention. The analysis methods simulate a CC experiment using a list of the target subsequences and a 35 database of the DNA sequences likely to be present in a sample to be analyzed. The simulation results in a hash code table which contains for each hash code all possible

sequences that can generate that code. Thereby, interpretation of a detected hash code requires a look-up in the table to find the possible sequences.

It is preferable that subsequences be carefully chosen in order that a minimum set of targets be obtained, preferably no more than approximately 20, that produce the maximum amount of information. Computer implemented methods of this invention determine optimum sets of target subsequences for a given database of sequences likely to occur in the sample by optimizing the number of non-empty hash codes in the simulated hash code table.

Maximum information is obtained when the target subsequences occur completely randomly in the possible sample sequences, that is, when their likelihood of occurrence is approximately 50% and the presence of one subsequence is independent of the presence of any other subsequence. Therefore, target subsequences chosen to generate a signal should preferably occur in the DNA sequence sample to be analyzed less than about 50% and at least more often than 5-10%, preferably more often than 10-15%. The most preferable occurrence probability is from 25-50%. Also the presence of one target subsequence is preferably probabilistically independent of the presence of any other subsequence.

Using data on expressed RNA from human DNA sequence databases, this means that sub-sequences are preferably less than about 5 to 8 bp long for cDNA classification.

Typically, the resulting preferable target subsequences are 4 to 6 bp long. Longer sequences occur too infrequently to be preferred for use. However, for classifying gDNA, longer subsequences, up to 20 to 40 bp, are preferably used, because gDNA fragments are normally of much greater length, from at least 5 kilobases ("kb") for plasmid inserts to more than 100 kb for P1 inserts, and thus would typically have more sequence variability, requiring longer target subsequences.

35 The preferred hybridization probes for short target subsequences are labeled peptido-nucleic acids (PNAs). Alternatively sets of degenerate, longer DNA oligonucleotides

are used which include as a common subsequence the target subsequence. These degenerate sets achieve improved hybridization specificity as compared to 4 to 6-mers. Sets of probes, each probe distinctively and distinguishably 5 labeled with a fluorochrome, are hybridized in conditions of high stringency to arrayed DNA sequence clones and optically detected to detect the presence of target subsequences. For example, in an embodiment wherein five fluorochromes are simultaneously distinguished and 20 subsequences observations 10 are required for gene identification (a 20 bit code), any gene in a colony can be identified in only four hybridization steps. Alternately, efficient hybridization detection means based on optical wave guide detection of DNA hybridization can be used. By using differently sized and shaped particles 15 associated with different probes, the resultant differences in light scattering can be used to detect hybridization of multiple probes simultaneously with these wave guide methods.

Target subsequences can be chosen to discriminate not only single genes but also, more coarsely, sets of genes. 20 Fewer target subsequences can be chosen so that a particular pattern of hits will indicate the presence of a gene of a particular type. Types of genes of interest might be oncogenes, tumor suppressor genes, growth factors, cell cycle genes, or cytoskeletal genes, etc.

25 In embodiments of this invention where high stringency hybridization are specified, such conditions generally comprise a low salt concentration, equivalent to a concentration of SSC (173.5 g. NaCl, 88.2 g. Na Citrate, H₂O to 1 l.) of less than approximately 1 mM, and a temperature 30 near or above the T_m of the hybridizing DNA. In contrast, conditions of low stringency generally comprise a high salt concentration, equivalent to a concentration of SSC of greater than approximately 150 mM, and a temperature below the T_m of the hybridizing DNA.

35 In embodiments of this invention where DNA oligomers are specified for performing functions, including hybridization and chain elongation priming, alternatively

oligomers can be used that comprise those of the following nucleotide mimics which perform similar functions.

Nucleotide mimics are subunits (other than classical nucleotides) which can be polymerized to form molecules capable of specific, Watson-Crick-like base pairing with DNA.

The oligomers can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof. The oligomers can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligomers may include other appending groups such as peptides, hybridization-triggered cleavage agents (see, e.g., Krol et al., 1988, *BioTechniques* 6:958-976), or intercalating agents (see, e.g., Zon, 1988, *Pharm. Res.* 5:539-549). The oligomers may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The oligomers may also comprise at least one nucleotide mimic that is a modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine. The oligomers may comprise at least one modified sugar moiety selected from the group including but

not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose. The oligomers may comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

The oligomer may be an α -anomeric oligomer. An α -anomeric oligomer forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, *Nucl. Acids Res.* 15:6625-6641).

Oligomers of the invention may be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligos may be synthesized by the method of Stein et al. (1988, *Nucl. Acids Res.* 16:3209), methylphosphonate oligos can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451), etc.

In specific embodiments of this invention it is preferable to use oligomers that can specifically hybridize to subsequences of a DNA sequence too short to achieve reliably specific recognition, such that a set of target subsequences is recognized. Further where PCR is used, as Tag polymerase tolerates hybridization mismatches, PCR specificity is generally less than hybridization specificity. Where such oligomers recognizing short subsequences are preferable, they may be constructed in manners including but not limited to the following. To achieve reliable hybridization to shorter DNA subsequences, degenerate sets of DNA oligomers may be used which are constructed of a total length sufficient to achieve specific hybridization with each member of the set containing a shorter sequence complementary to the common subsequence to be recognized. Alternatively, a longer DNA oligomer may be constructed with a shorter

sequence complementary to the subsequence to be recognized and with additional universal nucleotides or nucleotide mimics, which are capable of hybridizing to any naturally occurring nucleotide. Nucleotide mimics are sub-units which 5 can be polymerized to form molecules capable of specific, Watson-Crick-like base pairing with DNA. Alternatively, the oligomers may be constructed from DNA mimics which have improved hybridization energetics compared to naturally occurring nucleotides.

10 A preferred mimic is a peptido-nucleic acid ("PNA") based on a linked N-(2-aminoethyl)glycine backbone to which normal DNA bases have been attached (Egholm et al., 1993, *Nature* 365:566-67). This PNA obeys specific Watson-Crick base pairing, but with greater free energy of binding and 15 correspondingly higher melting temperatures. Suitable oligomers may be constructed entirely from PNAs or from mixed PNA and DNA oligomers.

20 In embodiments of this invention where DNA fragments are separated by length, any length separation means known in the art can be used. One alternative separation means employs a sieving medium for separation by fragment length coupled with a force for propelling the DNA fragments through the sieving medium. The sieving medium can be a polymer or gel, such as a polyacrylamide or agarose in 25 suitable concentrations to separate 10-1000 bp DNA fragments. In this case the propelling force is a voltage applied across the medium. The gel can be disposed in electrophoretic configurations comprising thick or thin plates or capillaries. The gel can be non-denaturing or denaturing. 30 Alternately, the sieving medium can be such as used for chromatographic separation, in which case a pressure is the propelling force. Standard or high performance liquid chromatographic ("HPLC") length separation means may be used. An alternative separation means employs molecular 35 characteristics such as charge, mass, or charge to mass ratio. Mass spectrographic means capable of separating 10-1000 bp fragments may be used.

DNA fragment lengths determined by such a separation means represent the physical length in base pairs between target subsequences, after adjustment for biases or errors introduced by the separation means and length changes 5 due to experimental variables (e.g., presence of a detectable label, ligation to an adapter molecule). A represented length is the same as the physical length between occurrences of target subsequences in a sequence from said database when both said lengths are equal after applying corrections for 10 biases and errors in said separation means and corrections based on experimental variables. For example, represented lengths determined by electrophoresis can be adjusted for mobility biases due to average base composition or mobility changes due to an attached labeling moiety and/or adapter 15 strand by conventional software programs, such as Gene Scan Software from Applied Biosystems, Inc. (Foster City, CA).

In embodiments of this invention where DNA fragments must be labeled and detected, any compatible labeling and detection means known in the art can be used. 20 Advances in fluorochromes, in optics, and in optical sensing now permit multiply labeled DNA fragments to be distinguished even if they completely overlap in space, as in a spot on a filter or a band in a gel. Results of several recognition reactions or hybridizations can be multiplexed in the same 25 gel lane or filter spot. Fluorochromes are available for DNA labeling which permit distinguishing 6-8 separate products simultaneously (Ju et al., 1995, *Proc. Natl. Acad Sci. USA* 92:4347-4351).

Exemplary fluorochromes adaptable to this invention 30 and methods of using such fluorochromes to label DNA are described in Sec. 6.11.

Single molecule detection by fluorescence is now becoming possible (Eigen et al., 1994, *Proc. Natl. Acad Sci. USA* 91:5740-5747), and can be adapted for use.

35 In embodiments of this invention where intercalating DNA dyes are utilized to detect DNA, any such dye known in the art is adaptable. In particular such dyes

include but are not limited to ethidium bromide, propidium iodide, Hoechst 33258, Hoechst 33342, acridine orange, and ethidium bromide homodimers. Such dyes also include POPO, BOBO, YOYO, and TOTO from Molecular Probes (Eugene, OR).

5 Finally alternative sensitive detection means available include silver staining of polyacrylamide gels (Bassam et al., 1991, *Analytic Biochemistry* 196:80-83), and the use of intercalating dyes. In this case the gel can be photographed and the photograph scanned by scanner devices 10 conventional in the computer art to produce a computer record of the separated and detected fragments. A further alternative is to blot an electrophoretic separating gel onto a filter (e.g., nitrocellulose) and then to apply any visualization means known in the art to visualize adherent 15 DNA. See, e.g., Kricka et al., 1995, *Molecular Probing, Blotting, and Sequencing*, Academic Press, New York. In particular, visualization means requiring secondary reactions with one or more reagents or enzymes can be used, as can any means employed in the CC embodiment.

20 A preferred separation and detection apparatus for use in this invention is found in copending U.S. Patent Application Serial No. 08/438,231 filed May 9, 1995, which is hereby incorporated by reference in its entirety. Other detection means adaptable to this invention include the 25 commercial electrophoresis machines from Applied Biosystems Inc. (Foster City, CA), Pharmacia (ALF), Hitachi, Licor. The Applied Biosystems machine is preferred among these as it is the only machine capable of simultaneous 4 dye resolution.

In the following subsections and the accompanying 30 examples sections QEA™ and the CC embodiments are described in detail.

5.1. QUANTITATIVE EXPRESSION ANALYSIS

This embodiment of this invention in the tissue 35 mode preferably generates one or more signals unique to each cDNA sequence in a mixture of cDNAs, such as may be derived from total cellular RNA or total cellular mRNA from a tissue

sample, and to quantitatively relate the strength of such a signal or signals to the relative amount of that cDNA sequence in the sample or library. In the query mode, this embodiment preferably generates signals uniquely 5 discriminating only a few sample sequences of interest in a quantitative manner. Less preferably, the signals uniquely determine only sets of a small number of sequences, typically 2 - 10 sequences. QEA™ signals comprise an indication of the presence of pairs of target subsequences and the length 10 between pairs of adjacent subsequences in a DNA sample. Alternatives include recognizing the presence of third subsequences between the pairs of target subsequences. In a further embodiment ("5'-QEA™"), one of the subsequences is 15 the true end of the protein coding sequence, in a defined relation to the 5' cap of the source mRNA. Signals are preferably generated in a manner permitting straightforward automation with existing laboratory robots. For simplicity of disclosure, and not by way of limitation, the detailed description of this method is directed to the analysis of 20 samples comprising a plurality of cDNA sequences. It is equally applicable to samples comprising a single sequence or samples comprising sequences of other types of DNA or nucleic acids generally.

While described in terms of cDNA hereinbelow, it 25 will be understood that the DNA sample can be cDNA and/or genomic DNA, and preferably comprises a mixture of DNA sequences. In specific embodiments, the DNA sample is an aliquot of cDNA of total cellular RNA or total cellular mRNA, most preferably derived from human tissue. The human tissue 30 can be diseased or normal. In one embodiment, the human tissue is malignant tissue, e.g., from prostate cancer, breast cancer, colon cancer, lung cancer, lymphatic or hematopoietic cancers, etc. In another embodiment, the tissue may be derived from in vivo animal models of disease 35 or other biologic processes. In this cases the diseases modeled can usefully include, as well as cancers, diabetes, obesity, the rheumatoid or autoimmune diseases, etc. In yet

another embodiment, the samples can be derived from *in vitro* cultures and models. This invention can also be advantageously applied to examine gene expression in plants, yeasts, fungi, etc.

5 The cDNA, or the mRNA from which it is synthesized, must be present at some threshold level in order to generate signals, this level being determined to some degree by the conditions of a particular QEA™ experiment. For example, such a threshold is that preferably at least 1000, and more 10 preferably at least 10,000, mRNA molecules of the sequence to be detected be present in a sample. In the case where one or only a few mRNAs of a type of interest are present in each cell of a tissue from which it is desired to derive the sample mRNA, at least a corresponding number of such cells 15 should be present in the initial tissue sample. In a specific embodiment, the mRNA detected is present in a ratio to total sample RNA of 1:10⁵ to 1:10⁶. With a lower ratio, more molecular amplification can be performed during a QEA™ experiment.

20 The cDNA sequences occurring in a tissue derived pool include short untranslated sequences and translated protein coding sequences, which, in turn, may be a complete protein coding sequence or some initial portion of a coding sequence, such as an expressed sequence tag. A coding 25 sequence may represent an as yet unknown sequence or gene or an already known sequence or gene entered into a DNA sequence database. Exemplary sequence databases include those made available by the National Center for Biotechnology Information ("NCBI") (Bethesda, MD) (GenBank) and by the 30 European Bioinformatics Institute ("EMBL") (Hinxton Hall, UK).

 A QEA™ method is also applicable to samples of genomic DNA in a manner similar to its application to cDNA. In gDNA samples, information of interest includes occurrence 35 and identity of translocations, gene amplifications, loss of heterozygosity for an allele, etc. This information is of interest in cancer diagnosis and staging. In cancer

patients, amplified sequences might reflect an oncogene, while loss of heterozygosity might reflect a tumor suppressor gene. Such sequences of interest can be used to select target subsequences and to predict signals generated by a 5 QEA™ experiment. Even without prior knowledge of the sequences of interest, detection and classification of QEA™ signal patterns is useful for the comparison of normal and diseased states or for observing the progression of a disease state. Gene expression information concerning the 10 progression of a disease state is useful in order to elucidate the genetic mechanisms behind disease, to find useful diagnostic markers, to guide the selection and observe the results of therapies, etc. Signal differences identify the gene or genes involved, whether already known or yet to 15 be sequenced.

Classification of QEA™ signal patterns, in an exemplary embodiment, can involve statistical analysis to determine significant differences between patterns of interest. This can involve first grouping samples that are 20 similar in one or more characteristics, such characteristics including, for example, epidemiological history, histopathological state, treatment history, etc. Signal patterns from similar samples are then compared, e.g., by finding the average and standard deviation of each individual 25 signals. Individual signals which are of limited variability, for which the standard deviation is less than the average, then represent genetic constants of samples of this particular characteristic. Such limited variability signals from one set of tissue samples can then be compared 30 to limited variability signals from another set of tissue samples. Signals which differ in this comparison then represent significant differences in the genetic expression between the tissue samples and are of interest in reflecting the biological differences between the samples, such as the 35 differences caused by the progression of a disease. For example, a significant difference in expression is detected with the difference in the genetic expression between two

tissues exceed the sum of the standard deviation of the expressions in the tissues. Other standard statistical comparisons can also be used to establish level of expression and the significance of differences in levels of expressions.

5 Target subsequence choice is important in the practice of this invention. The two primary considerations for selecting subsequences are, first, redundancy, that is, that there be enough target subsequence pair occurrences (also known as "hits") per gene that a unique signal is 10 likely to be generated for each sample sequence, and second, resolution, that is, that there not be so target subsequence pair occurrences with very similar lengths in a sample that the signals cannot be resolved. For sufficient redundancy, it is preferable that there be on average, approximately 15 three target subsequence pair hits per gene or DNA sequence in the sample. It is highly preferable that there be a minimum of at least one pair hit per each gene. In tests of a database of eukaryotic expressed sequences, it has been found that an average value of three pair hits per gene appears to 20 be generally a sufficient guarantee of this minimum criterion.

Sufficient resolution depends on the separation and detection means chosen. For a particular choice of separation and detection means, a recognition reaction 25 preferably should not generate more fragments than can be separated and distinguishably detected. In a preferred embodiment, gel electrophoresis is the separation means used to separate DNA fragments by length. Existing electrophoretic techniques allow an effective resolution of 30 three base pair ("bp") length differences in sequences of up to 1000 bp length. Given knowledge of fragment base composition, effective resolution down to 1 bp is possible by predicting and correcting for the small differences in mobility due to differing base composition. However and 35 without limitation, an easily achievable three bp resolution is assumed by way of example in the description of the invention herein. It is preferable for increased detection

efficiency that the distinguishably labeled products from as many recognition reactions as possible be combined for separation in one gel lane. This combination is limited by the number of labels distinguishable by the employed 5 detection means. Any alternative means for separation and detection of DNA fragments by length, preferably with resolution of three bp or better, can be employed. For example, such separation means can be thick or thin plate or column electrophoresis, column chromatography or HPLC, or 10 physical means such as mass spectroscopy.

The redundancy and resolution criteria are probabilistically expressed in Eqns. 1 and 2 in an approximation adequate to guide subsequence choice. In these 15 equations the number of genes in the cDNA sequence mixture is N , the average gene length is L , the number of target subsequence pairs is M (the number of pairs of recognition means), and the probability of each target subsequence occurring in, or hitting, a typical sample sequence is p . Since each target subsequences is preferably selected to 20 occur independently in each sample sequence, the probability of occurrence of an arbitrary subsequence pair is then p^2 . Eqn. 1 expresses the redundancy condition of three pair occurrences per sample sequence, assuming the probability of occurrence of each target subsequence is independent.

25

$$Mp^2 = 3 \quad (1)$$

Eqn 2 expresses the resolution condition of having fragments with lengths no closer on average than 3 base pairs. This 30 equation approximates the actual fragment length distribution with a uniform distribution.

$$\frac{L}{Np^2} = 3 \quad (2)$$

Given expected values of N , the number of sequences in the 35 library or sample to analyze (library complexity), and L , the average expressed sequence (or gene) length, Eqns 1 and 2 are

solved for the subsequence occurrence probability and number of subsequences required. This solution depends on the particular redundancy and resolution criteria dictated by the particular experimental method chosen to implement QEA™.

5 Alternative values may be required for other implementations of this embodiment.

For example, it is estimated that the entire human genome contains approximately 10^5 protein coding sequences with an average length of 2000. The solution of Eqns 1 and 2 10 for these parameters is $p = 0.082$ and $M = 450$. Thereby the expression of all genes in all human tissues can be analyzed with 450 target subsequence pairs, each subsequence having an independent probability of occurrence of 8.2%. In an embodiment in which eight fluorescently labeled subsequence 15 pairs can be optically distinguished and detected per electrophoresis lane, such as is possible when using the separation and detection apparatus described in copending U.S. Patent Application Serial No. 08/438,231 filed May 9, 1995, 450 reactions can be analyzed in only 57 lanes. 20 Thereby only one electrophoresis plate is needed in order to completely determine all human genome expression levels. Since the best commercial machines known to the applicants can discriminate only four fluorescent labels in one lane, a corresponding increase in the number of lanes is required to 25 perform a complete genome analysis with such machines.

As a further example, it is estimated that a typically complex human tissue expresses approximately 15,000 genes. The solution for $N = 15000$ and $L = 2000$ is $p = 0.21$ and $M = 68$. Thus expression in a typical tissue can be analyzed with 30 68 target subsequence pairs, each subsequence having an independent probability of occurrence of 21%. Assuming 4 subsequence pairs can be run per gel electrophoresis lane, the 68 reactions can be analyzed in 17 lanes in order to determine the gene expression frequencies in any human 35 tissue. Thus it is clear that this method leads to greatly simplified quantitative gene expression analysis within the capabilities of existing electrophoretic systems.

These equations provide an adequate guide to picking subsequence pairs. Typically, preferred probabilities of target subsequence occurrence are from approximately 0.01 to 0.30. Probabilities of occurrence of 5 specified subsequences and RE recognition sites can be determined from databases of DNA sample sequences. Example 6.2 lists these probabilities for exemplary RE recognition sites. Appropriate target subsequences can be selected from these tables. Computer implemented QEA™ experimental design 10 methods can then optimize this initial selection.

Another use of QEA™ is to compare directly the expression of only a few genes or sample sequences, typically 1 to 10, between two different tissues, the query mode, instead of seeking to determine the expression of all genes 15 in a tissue, the tissue mode. In this query mode, a few target subsequences are selected to discriminate the genes of interest both among themselves and from all other sequences possibly present. The computer design methods described hereinbelow can make this selection. If 4 subsequence pairs 20 are sufficient for identification, then the fragments from the 4 recognition reactions performed on each tissue are preferably separated and detected on two separate lanes in the same gel. If 2 subsequence pairs are sufficient for identification, the two tissues are preferably analyzed in 25 the same gel lane. Such comparison of signals from the same gel improves quantitative results by eliminating measurement variability due to differences between separate electrophoretic runs. For example, expression of a few target genes in diseased and normal tissue samples can be 30 rapidly and reliably analyzed.

The query mode of QEA™ is also useful even if the sequences of the particular genes of interest are not yet known. Differentially expressed features can be identified by comparing the results of QEA™ reactions applied to two 35 different samples. In the case where the separation and detection of reaction products is by gel electrophoresis, such a comparison can be done by comparing gel bands or

fluorescent traces of exiting fragments. Such differentially expressed features can then retrieved from the gel by methods known in the art (e.g., electro-elution from the gel) and the DNA fragments analyzed by conventional techniques, such as by sequencing. Such sequences, which are typically partial, can then be used as probes (e.g., in PCR or Southern blot hybridization) to recover full-length sequences. In this manner, QEA™ techniques can guide the discovery of new differentially expressed cDNA or of changes of the state of gDNA. The sequences of the newly identified genes, once determined, can then be used to guide QEA™ target subsequence choice for further analysis of the differential expression of the new genes.

Alternative embodiments of QEA™ are described herein, differing primarily in how the recognition means recognize the target subsequences. Associated with these primary differences are secondary differences in how signals are generated from the recognition means. In the PCR embodiment, target subsequences are recognized by oligomers which hybridize to the DNA target subsequences and act as PCR primers for the amplification of the segments between adjacent primer pairs. Amplified fragments from a sample are separated preferably by electrophoresis. Selection of target subsequences, the primer hybridizing sites, meeting the probability of occurrence and independence criteria is preferably made from a database containing sequences expected to be present in the samples to be analyzed, for example human GenBank sequences, and optimized by the computer implemented experimental design methods. In a preferred embodiment, subsequence selection begins by compiling oligomer frequency tables containing the frequencies of, preferably, all 4 to 8-mers by using a sequence database. From these tables, target subsequences with the necessary probabilities of occurrence according to Eqns. 1 and 2 are selected and checked for independence, by, for example, checking that the conditional probability for occurrence by any selected pair of subsequences is the product of the

probabilities of occurrences of the individual subsequences of the pair. An initial selection can be optimized to determine target subsequence sets producing unique fragments from the greatest number of sample sequences. PCR primers 5 are synthesized with a 3' end complementary to the chosen subsequences and used in the PCR embodiment. Example 6.1 illustrates the signals output by this method in a specific example.

The preferred embodiment uses DNA binding proteins, 10 specifically REs, including Type IIS REs, to recognize and cleave sample sequences at the target subsequences. Desired fragments, with lengths dependent only on source cDNA sequence, are amplified by an amplification means in order to dilute remaining, unwanted fragments with indefinite lengths. 15 Typically, but without limitations, desired fragments are doubly cut by REs whereas unwanted fragments are singly cut. But in 5'-QEA™, singly cut fragments have a definite length and are of interest. Unwanted singly cut fragments can be removed by affinity means (e.g., biotin labeling), physical 20 means (e.g., hydroxyapatite column separation), or enzymatic means (e.g., single strand specific nucleases). Sufficient removal of the unwanted singly cut ends from the desired doubly cut fragments can permit fragment detection without an amplification step. For the RE alternative embodiments, the 25 possible target subsequences, although limited to recognition sites of available REs, can be selected in a manner similar to the above in order to meet the previous probability or occurrence and independence criteria as closely as possible. For example, the probabilities of occurrence of various RE 30 recognition sites can be determined from a database of potential sample sequences, and those REs chosen with recognition subsequences whose probabilities of occurrence meet the criterion of Eqns 1 and 2 as closely as possible. If multiple REs satisfy the selection criteria, a subset is 35 selected by including only those REs with independently occurring recognition subsequences, determined, for example, in the previous manner using conditional probabilities of

occurrence. An initial choice can be optionally optimized by the computer implemented experimental design methods.

A number, R_e , of REs are preferably selected so that the number of RE pairs is approximately M , as determined from 5 Eqn. 1, where the relation between M and R_e is given by Eqn. 3.

$$M = \frac{R_e(R_e + 1)}{2} \quad (3)$$

¹⁰ For example, a set of 20 acceptable REs results in 210 subsequence pairs.

There are numerous REs currently available, whose recognition sequences have a wide range of occurrence probabilities, from which REs can be selected for the present ¹⁵ invention. Exemplary REs are listed in Sec. 6.2.

The PCR and the RE embodiments have different accuracy and flexibility characteristics. RE embodiments are generally more accurate, with fewer false positive and false ²⁰ negative identifications, since the enzymatic recognition and subsequent ligation reactions are generally more specific than the hybridization of short PCR primers to their subsequence targets, even under stringent hybridization conditions.

²⁵ Restriction endonucleases ("RE") generally bind with specificity only to their four to eight bp recognition sites, cleaving the DNA preferably with an at least 2 bp overhang. Although it is preferable that REs used produce overhangs of known sequence and characteristic of the particular RE, other REs, such as those known as class IIS ³⁰ restriction enzymes, which produce overhangs of unknown sequence can be used to extend initial target subsequences into longer effective target subsequences. Phasing primers can also be used to recognize longer effective target ³⁵ subsequences. Overhangs of the initial REs can be specifically recognized by hybridization of an adapter followed by ligation of one strand of this adapter, the

amplification primer. The ligase enzymes, which are used in this alternative embodiment of this invention to ligate the amplification primer, are highly specific in their hybridization requirements; even one bp mismatch near the 5 ligation site will prevent ligation (U.S. Patent 5,366,877, Nov. 22, 1994, to Keith et al.; U.S. Patent 5,093,245, Mar. 3, 1992, to Keith et al.). On the other hand, PCR and the preferred Taq polymerase used therein tolerates hybridization mis-matches of elongation primers. Thus, PCR embodiments can 10 generate false positive signals which arise from mis-matches in the hybridization of the oligomer probes to the target subsequences. However, the PCR embodiments are more flexible since any desired subsequence can be a target subsequence. The RE embodiment is limited to the recognition sequences of 15 acceptable REs. However, more than 150 to 200 REs are now commercially available recognizing a wide variety of nucleotide sequences.

QEA™ experiments are also adaptable to distinguish sample sequences into small sets, typically comprising 2 to 20 10 sequences. Such coarser grain analysis requires fewer subsequence pairs, fewer recognition reactions, and less analysis time. Alternatively, smaller numbers of target subsequence pairs can be optimally chosen to distinguish individually a specific set of sequences of interest from all 25 the other sequences in a sample. These target subsequences can be chosen either from REs that produce fragments from the specific sample sequences or, in the case of the PCR embodiment, from a set of subsequences optimized for this specific set of sequences.

30 Detailed descriptions of exemplary implementations for practicing QEA™ recognition reactions and the computer implemented experimental analysis and design methods are presented in the following subsections. Detailed experimental protocols appear in Sec. 6. These 35 implementations are illustrative and not limiting, as this embodiment of the invention may be practiced by any method generating the previously described QEA™ signals.

5.2. RE EMBODIMENTS OF QEA™

The preferred restriction endonuclease ("RE") embodiments of QEA™ use novel simultaneous RE and ligase enzymatic reactions, known as recognition reactions, for generating labeled fragments of the sample sequences to be analyzed. These labeled fragments are then optionally amplified by an amplification means, separated according to length by a separation means, and detected by a detection means to yield QEA™ signals comprising the identity of the REs cutting each fragment together with each fragment's length. The RE/ligase subsequence recognition reactions can specifically and reproducibly generate QEA™ signals with good signal to noise ratios. Preferred protocols for this reaction perform all steps, including amplification, in a single tube without any intermediate extractions or buffer exchanges. This protocol is preferably automatically performed by standard laboratory robots.

REs bind with specificity to short DNA target subsequences, usually 4 to 8 bp long, that are termed "recognition sites" and are characteristic of each RE. REs that are used cut the sequence at (or near) these recognition sites preferably producing characteristic ("sticky") ends with single-stranded overhangs, which usually incorporate part of the recognition site. Type IIS REs, which cut outside of their recognition site, can be used to extend the initial target subsequence to a longer effective target subsequence for use in the computer implemented database lookup.

Preferred REs have a 6 bp recognition site and generate a 4 bp 5' overhang. Less preferred REs generate a 2 bp 5' overhang. These are less preferred since 2 bp overhangs have a lower ligase substrate activity than 4 bp overhangs. All RE embodiments can be adapted to 3' overhangs of two and four bp. In order that an amplification primer hybridization site can be presented on each of the two strands of the product of the RE/ligase recognition reaction, as is necessary for experimental amplification. REs

generating 5' and 3' overhangs are preferably not used in the same recognition reaction. Further, preferred REs have the following additional properties. Their recognition sites and overhang sequences are preferably such that an amplification 5 primer can be designed whose ligation does to a cut end does not recreate the recognition site. They preferably have sufficient activity below 37°C, and particularly at 16°C, the optimal ligase temperature, to cut unwanted ligation products, and are heat inactivated at 65°C and above so that 10 PCR amplification can be performed by simply adding PCR reagents to the RE/ligase reaction mix. They preferably have low non-specific cutting and nuclease activities and cut to completion. The REs selected for a particular experiment 15 preferably have recognition sites meeting the previously described occurrence and independence criteria. Preferred pairs of REs for analyzing human and mouse cDNA are listed in Sec. 6.10.

Only cDNA fragments with definite and reproducible lengths dependent only on the source cDNA sequence and 20 independent of cDNA synthesis conditions are of interest. Only such fragments of definite length are adaptable to the experimental analysis methods in order to determine their originating sample sequence. cDNA fragments doubly cut on each end and by REs have a length dependent only on the 25 sequence of the originating cDNA and are, therefore, of interest. cDNA fragments singly cut on their 5' end by an RE and terminated on their 3' end by the poly(A) tail have a variable and non-reproducible lengths that depend strongly on cDNA synthesis conditions. Such fragments singly cut on one 30 end by an RE and with a variable length tail on the other are not of interest. To separate signals from doubly cut fragments from the unwanted signals from singly cut fragments, certain RE embodiments of QEA™ exponentially amplify doubly cut fragments, while only linearly amplifying 35 singly cut fragments. This amplification is preferably done by the PCR method. Other RE embodiments separate singly and doubly cut fragments with a removal means targeted at either

type of fragment. The preferred removal means comprises a biotin capture moiety and a streptavidin binding partner. The removal means can either supplement or replace differential amplification. On the other hand, cDNA 5 fragments singly cut on their 3' end by an RE and terminated on their 5' end by a sequence in a fixed relation to the 5' cap of the source mRNA also have definite lengths and are of interest. Such fragments can be generated according to a method herein called 5'-QEATM, which comprises synthesizing 10 cDNA according to the protocol of Sec. 6.3.3, performing recognition reactions, and separating the fragments of interest by a removal means. Alternatively, fragments are also of interest if they have a definite, sequence dependent length by being singly cut on their 5' end and by being 15 terminated in a fixed relation with respect to the beginning of the 3' poly(A)+ tail.

This invention is adaptable to alternative amplification means known in the art. If a removal means for unwanted singly cut fragments is not utilized, alternative 20 amplification means must preferentially amplify doubly cut fragments with respect to singly cut fragments, in order that signals from singly cut fragments be relatively suppressed. On the other hand, if a removal means for singly cut fragments is utilized in an embodiment, then alternative 25 amplification means can less preferably have no amplification preference. In RE embodiments using a removal means, this means can be used either to remove the singly or the doubly cut fragments. Known alternative amplification means are listed in Kricka et al., 1995, Molecular Probing, Blotting, 30 and Sequencing, chap. 1 and table IX, Academic Press, New York. Of these alternative means, those employing the T7 RNA polymerase are preferred.

Certain other embodiments use a physical removal means to directly remove unwanted singly cut fragments, 35 preferably before amplification. Singly cut fragment removal can be accomplished, e.g., by labeling DNA termini with a capture moiety prior to digestion, as by synthesizing the

1 cDNA with biotinylated primers. After digestion, the singly
2 cut fragments are then removed by contacting the sample with
3 a binding partner of the capture moiety, affixed to a solid
4 phase. Alternatively, the doubly cut fragments can be
5 labeled with a capture moiety, as by amplifying the fragments
6 with primers one of which is labeled with a capture moiety.
7 The amplification products are contacted with a binding
8 partner affixed to a solid support, washed, and then
9 denatured. Thereby, only doubly cut fragments, one end of
10 which is labeled with a capture moiety, are separated.
11 Alternately, single stranded fragments can be removed by
12 single strand specific column separation or single strand
13 specific nucleases.

14 This invention is applicable to any removal means
15 meeting the following minimal requirements. The removal
16 means includes a capture moiety and a binding partner. The
17 capture moiety is capable of conjugation to DNA oligomers
18 without disruption of hybridization or chain elongation
19 reactions. The binding partner is capable of attachment to a
20 solid phase support and can bind the capture moiety to such a
21 support in DNA denaturing conditions. The preferred removal
22 means is biotin-streptavidin. Other removal means adaptable
23 to this invention include various haptens, which are removed
24 by their corresponding antibodies. Exemplary haptens include
25 digoxigenin, DNP, and fluorescein (Holtke et al., 1992,
26 Sensitive chemiluminescent detection of digoxigenin labeled
27 nucleic acids: a fast and simple protocol for applications,
28 *Biotechniques* 12(1):104-113 and Olesen et al., 1993,
29 Chemiluminescent DNA sequencing with multiple labeling,
30 *Biotechniques* 15(3):480-485).

31 RE/ligase embodiments of QEA™ use recognition
32 moieties. In any one recognition reaction, each recognition
33 moiety is capable of hybridizing with and being ligated to
34 overhangs cut by only one RE. Thereby, the recognition
35 sequence of that RE is identified. Recognition moieties
36 typically comprise partially double stranded DNA oligomers,
37 each oligomer capable of specifically hybridizing with only

one RE generated sticky end in one recognition reaction. In the RE/ligase embodiment using PCR amplification, the recognition moieties also provide primer means for the PCR and thereby also provide for labeling and recognition of RE 5 cut ends. For example, using a pair of REs in one recognition reaction generates doubly cut fragments some with the recognition sequence of the first RE on both ends, some with the recognition sequence of the second RE on both ends, and the remainder with one recognition sequence of each RE on 10 either end. Using more REs generates doubly cut fragments with all pair-wise combinations of RE cut ends from adjacent RE recognition sites along the sample sequences. All these cutting combinations need preferably to be distinguished, since each provides unique information on the presence of 15 different subsequences pairs, the RE recognition sites, present in the original cDNA sequence. Thus the recognition moieties preferably have unique labels which label specifically each RE cut made in a reaction. As many REs can be used in a single reaction as labeled recognition moieties 20 are available to uniquely label each RE cut. If the detectable labeling in a particular system is, for example, by fluorochromes, then fragments cut with one RE have a single fluorescent signal from the one fluorochrome associated with that RE, while fragments cut with two REs 25 have mixed signals, one from the fluorochrome associated with each RE. Thus all possible pairs of fluorochrome labels are preferably distinguishable. Alternatively, if certain target subsequence information is not needed, the recognition moieties need not be distinctively labeled. In embodiments 30 using PCR amplification, corresponding primers would not be labeled. If silver staining is used to recognize fragments separated on an electrophoresis gel, no recognition moiety need be labeled, as fragments cut by the various RE combinations are not distinguishable.

35 The recognition reaction conditions are preferably selected, as described in Sec. 6.4, so that RE cutting and recognition moiety ligation go to full completion: all

recognition sites of all REs in the reaction are cut and ligated to a recognition moiety. In this manner, the fragments generated from a sequence analyzed lie only between adjacent recognition sites of any RE in that reaction. No 5 fragments remain which include an internal RE recognition site. Multiple REs can be used in one recognition reaction. Too many REs in one reaction can cut the sequences too frequently, generating a compressed length distribution with many short fragments of lengths between 10 and a few hundred 10 base pairs long that are not clearly resolvable by the separation means. For example, for gel electrophoresis, if the fragments are too close in length, fragments should not be closer than 3 bp on the average. Too many REs also can generate fragments of the same length and end subsequences 15 from different sample sequences. Finally, where fragment labels are to be distinguished, no more REs can be used than can have distinguishably labeled sticky ends. These considerations limit the number of REs optimally useable in one recognition reaction. Preferably two REs are used, with 20 one, three and four REs less preferable. Preferable pairs of REs for the analysis of human cDNA samples are listed in Sec. 6.10.

An additional level of sample sequence discrimination is possible by detecting occurrences of 25 internal subsequences (here called "third target subsequences"). The presence or absence of a third internal subsequences can be used in the computer implemented experimental analysis methods of this invention along with identification of the two end subsequences and the fragment 30 length to further discriminate the origin of otherwise identical fragment signals.

Fragments with specific third internal subsequences can be detected by either labeling or suppressing such fragments or with Type IIS REs. To label fragments with a 35 third internal subsequence, probes with distinguishable labels which bind to this target subsequence are added to the fragments prior to detection, and alternatively prior to

separation and detection. On detection, fragments with this third subsequence present will generate a signal, preferably fluorescent, from the probe. Such a probe could be a labeled PNA or DNA oligomer. Short DNA oligomers may need to be 5 extended with a universal nucleotide or degenerate sets of natural nucleotides in order to provide for specific hybridization. Fragments with a third subsequence can be suppressed in various manners. The absence of such fragments is determined by comparing a recognition reaction without the 10 suppressing factors with a reaction with the suppressing factors. First, in embodiments using PCR amplification, a probe hybridizing with this third subsequence which prevents polymerase elongation in PCR can be added prior to amplification. Then sequences with this subsequence will be 15 at most linearly amplified and their signal thereby suppressed. Such a probe could be a PNA or modified DNA oligomer (with the 3' nucleotide being a ddNTP). Second, if the third subsequence is recognized by an RE, this RE can be added to the RE-ligase reaction without any corresponding 20 specific primer. Fragments with the third subsequence thereby have primers on one end only are at most linearly amplified. Both these embodiments can be extended to multiple internal sequences by using multiple probes to recognize the sequences or to disrupt exponential PCR 25 amplification. Type IIS REs which cut a primer close to its junction with the original cDNA fragment sequence generates overhangs which are not contiguous with the initial RE recognition sequence. The sequence of such an overhang can be used as a third internal subsequence.

30

5.2.1. RECOGNITION MOIETY STRUCTURE

Construction of the recognition moieties, also herein called adapters or linker-primer oligomers, is important and is described here in advance of further details 35 of the individual recognition reaction steps. Their basic structure is first described, followed second by descriptions of several enhancements adaptable to QEA™ variations. In the

preferred embodiment, the adapters are partially double stranded DNA ("dsDNA"). Alternatively, the adapters can be constructed as oligomers of any nucleic acid having properties corresponding to those of the preferred DNA polymers. In an embodiment employing an alternative amplification means, the adapters preferably serve as a primer for that amplification means, if needed.

Turning first to basic adapter structure, Fig. 2A illustrates the DNA molecules involved in the ligation reaction as conventionally indicated with the 5' ends of the top strands and the 3' ends of the bottom strands at left. dsDNA 201 is a fragment of a sample cDNA sequence with an RE cut at the left end generating, preferably, four bp 5' overhang 202. Adapter dsDNA 209 is a synthetic substrate provided by this invention. The structure of adapter 209 is selected to ensure that RE digestion and adapter ligation preferably go to completion, that generation of unwanted products and amplification biases are minimized, and that unique labels are attached to cut ends (if needed). Adapter 209 comprises strand 203, called a primer, and a partially complementary strand 205, called a linker. The primer is also known as the longer strand of the adapter, and the linker is also known as the shorter strand of the adapter.

The linker, or shorter strand, links the cDNA cut by an RE to the primer, or longer strand, by hybridizing to the overhang generated by the RE and to the primer such that the 3' end of the primer is adjacent to the 5' end of the overhang. In this configuration, the primer can be effectively ligated to the cut dsDNA. Therefore, linker 205 comprises subsequence 206 complementary to RE overhang 202 and subsequence 207 complementary to 3' end 204 of primer 203. Subsequence 206 is most preferably of the same length as the RE overhang. Subsequence 207 is preferably eight nucleotides long, less preferably from 4 to 12 nucleotides long, but can be of any length as long as the linker reliably hybridizes with only one primer in any one recognition reaction at an appropriate T_m . The appropriate T_m should

preferably be less than the self-annealing T_m of primer 203. This ensures that subsequent PCR amplification conditions can be controlled so that linkers present in the reaction mixture will not hybridize and act as PCR primers, and, thereby, 5 generate spurious fragment lengths. The preferable T_m is less than approximately 68°C. Also, linker 205 preferably lacks a 5' terminal phosphate to prevent its ligated to the 3' bottom strand of dsDNA 201. More importantly, lack of a terminal phosphate also prevents self-annealed adapters from 10 ligating and forming dimers. Adapter self-ligation is disadvantageous in that it would compete with adapter ligation to cut cDNA fragments. Further, adapter dimers would be amplified in a subsequent amplification step generating unwanted fragments, termed amplification noise. 15 Terminal phosphates can be removed from linkers using phosphatases known in the art, followed by separation of the enzyme. An exemplary protocol for an alkaline phosphatase reaction is found in Sec. 6.3.4.

Primer, or longer strand, 203 has a 3' end 20 subsequence 204 complementary to 3' end subsequence 207 of linker 205. It is preferable that each RE generated overhang is ligated to a unique primer, in each recognition reaction in order that the overhangs generated by each RE can be detected. Consequently, in each recognition reaction primers 25 and linkers are preferably chosen so that each primer is complementary to and hybridizes with only one linker 205 and that each linker which hybridizes with an RE has a unique sequence 207 for hybridizing with a unique primer. In order that the primer/cDNA overhang ligation reaction go to 30 completion, primer 203 preferably does not recreate the recognition sequence of any RE in one recognition reaction when it is ligated with cDNA end 202. Further, primer 203 preferably has no 5' terminal phosphate in order to prevent primer self-ligations. To minimize amplification noise, it 35 is preferred that primer 203 not hybridize with any sequence present in the original sample mixture. If such hybridization occurred, a subsequence PCR step can amplify

unwanted fragments not cut by the initial REs. The T_m of primer 203 is preferably high, in the range from 50° to 80°C, and more preferably above 68°C. This permits that the subsequent PCR amplification can be controlled so that only 5 primers and not linkers initiate new chains, the linkers remaining melted through the PCR cycle. In the case of gel electrophoretic fragment separation and detection with, e.g. Ag staining or an intercalating dye, the primer is optionally unlabeled. For example, this T_m can be achieved by use of a 10 primer having a combination of a G+C content preferably from 40-60%, most preferably from 55-60%, and a length most preferably 24 nucleotides, and preferably from 18 to 30 nucleotides. Primer 203 is optionally labeled with fluorochrome 208, although any DNA labeling system that 15 preferably allows multiple labels to be simultaneously distinguished is usable in this invention. Generally, the primer, or longer strand, is constructed so that, preferably, it is highly specific, free of dimers and hairpins, and capable of forming stable duplexes under the conditions 20 specified, in particular at the desired T_m . Software packages are available for primer construction according to these principles, an example being OLIGO™ Version 4.0 For Macintosh from National Biosciences, Inc. (Plymouth, MN). In particular, a formula for T_m can be found in the OLIGO™ 25 Reference Manual at Eqn. I, page 2.

Fig. 2B illustrates two exemplary adapters and their component primers and linkers constructed according to the above description. Adapter 250 is specific for the RE BamHI, as it has a 3' end complementary to the 5' overhang 30 generated by BamHI. Adapter 251 is similarly specific for the RE HindIII. Sec. 6.10 contains a more comprehensive, non-limiting list of adapters that can be used according to the invention. All synthetic oligonucleotides of this invention are preferably as short as possible for their 35 functional roles in order to minimize synthesis costs. A further alternative illustrated in Fig. 2C is to construct an adapter by self hybridization of single stranded DNA in

hairpin loop configuration 212. Subsequences of loop 212 are constructed with similar structure to the corresponding subsequences of linker 205 and primer 203. Exemplary hairpin loop 211 sequences are C₄ to C₁₀.

5 REs generating 3' overhangs are less preferred and require different adapter structures. A preferable basic adapter structure for 3' overhangs is illustrated in Fig. 3A. dsDNA 301 is a fragment of a sample cDNA cut with a RE generating 3' overhang 302. Adapter 309 comprises primer, or 10 longer strand, 304 and linker, or shorter strand, 305. Primer, or longer strand, 304 includes subsequence 306 complementary to and of the same length as 3' overhang 302 and subsequence 307 complementary to linker 305. It also optionally has label 308 which distinctively labels primer 15 304. As in the case of adapters for 5' overhangs, in order that the RE digestion and ligation reactions go to completion, primer 304 preferably has no 5' terminal phosphate, in order to prevent self-ligations, and preferably has a sequence such that no recognition site for any RE in 20 one recognition reaction is created upon ligation of the primer with dsDNA 301. To minimize amplification, noise, primer 304 should preferably not hybridize with any sequence in the initial sample mixture. The T_m of primer 304 is preferably high, in the range from 50° to 80°C, and more 25 preferably above 68°C. This ensures the subsequent PCR amplification can be controlled so that only primers and not linkers initiate new chains. For example, this T_m can be achieved by using a primer having a G+C content preferably from 40-60%, most preferably from 55-60%, and a primer length 30 most preferably of 24 nucleotide and less preferably of 18-30 nucleotides. Each primer 304 in a reaction can optionally have a distinguishable label 308, which is preferably a fluorochrome.

Linker, or shorter strand, 305 is complementary to 35 and hybridizes with subsequence 307 of primer 304 in a position adjacent to 3' overhang 302. Linker 305 is most preferably 8 nucleotides long, less preferably from 4-16

nucleotides, and has no terminal phosphates to prevent self-ligation. This linker only promotes ligation specificity and activity and does not link primer 304 to the cut dsDNA, as in the 5' case. Further, linker 305 T_m should preferably be 5 less than primer 304 self-annealing T_m . This insures that subsequent PCR amplification conditions can be controlled so that linkers present in the reaction mixture will not hybridize and act as PCR primers, and, thereby, generate spurious fragment lengths. Fig. 3B illustrates an exemplary 10 adapter with its primer and linker for the case of the RE NlaIII. As in the 5' overhang case, a 3' adapter can also be constructed from a hairpin loop configuration.

Next, several adapter structural enhancements are described. The use of these enhancements is detailed in the 15 subsequent protocol descriptions. In one alternative, the adapter primer strand can have a conjugated capture moiety in addition to or in place of a conjugated label moiety. Such a label moiety is advantageous in separating various classes of RE/ligase reaction products by binding the capture moiety to 20 its binding partners. Acceptable and preferred capture moieties and binding partners have been previously described. Further, when a primer has a conjugated capture moiety, particularly biotin which form a streptavidin complex that is difficult to dissociate, it can advantageous to include a 25 release means in the primer in order to achieve controlled release from the bound capture moiety. Release means can involve including subsequences in the primer which can be cleaved in a controlled manner. One exemplary such subsequence is one or more uracil nucleotides. In this case 30 digestion with uracil DNA glycosylase (UDG) and subsequent hydrolysis of the sugar backbone at an alkaline pH effects releases. Another exemplary such subsequence is the 35 recognition subsequence of an RE which cuts extremely rarely if at all in the sequences of the sample. A preferred RE of this sort for human cDNA sequences is AscI, which has an 8 bp recognition sequence that rarely, if ever, occurs in mammalian DNA. AscI is further advantageously active at the

ends of DNA molecules. In this case, digestion with this RE, i.e., *Asc*I, will release strand 2351.

In another enhancement, adapters can be constructed from hybrid primers which are designed to facilitate the direct sequencing of a fragment or the direct generation of RNA probes for *in situ* hybridization with the tissue of origin of the DNA sample analyzed. Hybrid primers for direct sequencing are constructed by ligating onto the 5' end of existing primers the M13-21 primer, the M13 reverse primer, or equivalent sequences. Fragments generated with such hybrid adapters can be removed from the separation means and amplified and sequenced with conventional systems. Such sequence information can be used both for a previously known sequence to confirm the sequence determination and for a previously unknown sequence to isolate the putative new gene. Hybrid primers for direct generation of RNA hybridization probes are constructed by ligating onto the 5' end of existing primers the phage T7 promoter. Fragments generated with such hybrid adapters can be removed using the separation means and transcribed into anti-sense RNA with conventional systems. Such probes can be used for *in situ* hybridization with the tissue of origin of the DNA sample to determine in precisely what cell types a signal of interest is expressed. Such hybrid adapters are illustrated in Sec. 6.8.

In a further enhancement, the previously described adapters are used but the PCR primers strands have a extra subsequence 3' to the adapter primer strands in order to act as phasing primers. That is the PCR amplification reaction is used to recognize additional nucleotides beyond the initial RE target recognition subsequence. Fig. 2D illustrates such alternative phasing primers. In that figure, sample dsDNA 201 is illustrated after blunt-ending RE/ligase reaction products but just prior to a PCR amplification cycle. dsDNA 201 has been cleaved at position 221 producing overhang 202 by an RE recognizing target recognition subsequence 227, has been ligated to adapter primer strand 203, and has been completed to a blunt ended

double strand by strand 220 by incubation at 72°C for 10 minutes. For definiteness and without limitation, the RE recognition subsequence 227 typically extends 1 bp beyond overhang 202. Other relative positions depend on the lengths 5 of the overhang and the recognition sequence. Alternative PCR phasing primer 222, illustrated with its 5' end at the left, comprises subsequence 223, with the same sequence as strand 203; subsequence 224, with the same sequence as the RE overhang 202; subsequence 225, with a sequence consisting of 10 a remaining portion of RE recognition subsequence 227, if any; and subsequence 226 of P nucleotides. Length P is preferably from 1 to 6 and more preferably either 1 or 2. Subsequences 223 and 224 hybridize for PCR priming with corresponding subsequences of dsDNA 201. Subsequence 225 15 hybridizes with any remaining portion of recognition subsequence 227, typically 1 bp. Subsequence 226 hybridizes only with fragments 201 having complementary nucleotides in corresponding positions 228. When P is 1, PCR primer 222 selects for PCR amplification 1 of 4 possible fragments 201; 20 when P is 2, 1 of 16 are selected. Using 4 (or 16) primers 222, each with one of the possible (pairs of) nucleotides, in 4 (16) aliquots or RE/ligase reaction products selects for amplification one of the possible fragments 201. These primers are similar to phasing primers (European Patent 25 Application No. 0 534 858 A1, published Mar. 31, 1993).

The effect of using PCR primers 222, having subsequences 226 of length P bp, is to extend the initially recognized RE target subsequence into an effective target subsequence, which is the initial RE target subsequence 30 concatenated to a subsequence complementary to subsequence 226. Thereby, many additional target subsequences can be recognized while retaining the specificity and exactness characteristic of the RE embodiment. For example, REs recognizing 4 bp subsequences can be used in such a combined 35 reaction with an effective 5 or 6 bp target subsequence, which need not be palindromic. REs recognizing 6 bp sequences can be used in a combined reaction to recognize 7

or 8 bp sequences. Such effective recognition sequences are input to the computer implemented design and analysis methods subsequently described.

In a further enhancement, additional subsequence 5 information can be generated from adapters comprising primers with specially placed Type IIS RE recognition subsequence followed by digestion with the Type IIS RE and sequencing of the generated overhang. In a preferred embodiment, the Type IIS recognition subsequence is placed so that the generated 10 overhang is contiguous with the original recognition subsequence of the RE that cut the end to which the adapter hybridizes. In this embodiment, an effective target subsequence is formed by concatenating the sequence of the Type IIS overhang and the original recognition sequence. In 15 another embodiment, the Type IIS recognition sequence is placed so that the sequence of the generated overhang is not contiguous with the original recognition sequence. Here, the sequence of the overhang is used as a third internal subsequence in the fragment. In both cases, the additionally 20 recognized subsequence is used in the computer implemented experimental analysis methods to increase the capability of determining the source sequence of a fragment. This enhancement is illustrated in Figs. 17A-E and is described in detail in Sec. 5.2.3 ("The SEQ-QEA™ Embodiment"). The 25 primers used in the SEQ-QEA™ embodiment advantageously included combined enhancements, including label moieties, capture moieties, and release means.

It will be apparent to those of skill in the art that the previously described primers and linkers can be 30 enhanced with combinations of the previously described embodiment and with other alternatives known in the art to practice further embodiments and refinements of the RE/ligase embodiment of QEA™. This invention comprises these substantially similar variations of the embodiments described 35 herein.

5.2.2. RE/LIGASE METHOD STEPS

The steps of the preferred RE/ligase embodiment of QEA™ comprise: first, in one reaction cutting a cDNA sample with one or more REs, hybridizing adapters corresponding to the RES, and ligating the primers of the adapters on the cut 5 ends; second, amplifying the cut fragments, if necessary; and third, separating the fragments according to length and detecting fragment lengths and fragment target end subsequences. If necessary, prior to the first step, the cDNA sample can be synthesized by methods commonly known in 10 the art, such as those described in Sec. 6.3. Optionally, following the amplification step, additional steps to remove unwanted DNA fragments or RE/ligase reaction products prior to separation detection can increase QEA™ signal to noise ratio or simplify interpretation of the resulting signals. 15 Additional Re/ligase embodiments are described, including those known as 5'-QEA™ and SEQ-QEA™.

In more detail, the RE/ligase embodiment can begin with pre-synthesized cDNA, or with a tissue sample or mRNA from which cDNA is to be synthesized. When cDNA is to be 20 synthesized, the exemplary methods and procedures of Sec. 6.3 can be used. QEA™ does not require cloning into a vector. In the case of a tissue sample, a first step is the largely conventional separation of RNA from the tissue sample. Separated RNA is preferably poly(A)+ purified RNA, mRNA 25 separated from particular cellular fractions, or less preferably total cellular RNA. The steps of separation involve RNase extraction, DNase treatment and mRNA purification according to protocols, e.g., of Sec. 6.3.1. First and second strand cDNA synthesis from mRNA can be 30 performed according to the protocols of Sec. 6.3.2, or the less preferred protocols of Sec. 6.3.4. In the case of small quantities of mRNA or where it is advantageous to have full-length cDNA including complementary sequences out to the 5' cap of the source mRNA, the preferred synthesis protocols of 35 Sec. 6.3.3, or functionally equivalent protocols, can be used.

However obtained, it is important that cDNA used in the RE/ligase embodiment of QEA™ not have any terminal phosphates. This is to minimize noise in subsequent fragment length separation and detection caused by exponential amplification of unwanted fragments singly cut on one end by an RE and terminated on the other by a variable length oligo(dT) tail. Significant background noise can arise from exponential amplification of singly cut fragments whose blunt ends have ligated to form a single dsDNA with two cut ends having ligated primers, an apparently doubly cut fragment. The lengths of such fragment vary depending on cDNA synthesis conditions and produce diffuse background noise on gel electrophoresis, which obscures sharp bands from the normally doubly cut fragments. This background can be eliminated by preventing blunt end ligation of such singly cut cDNA fragments by initially removing all terminal phosphates from the cDNA sample, without otherwise disrupting the integrity of the cDNA. Thus, the final preparation step of a DNA sample is removal of terminal phosphates from the cDNA sample, if needed.

Thus the final preparation step of a cDNA sample is removal of terminal phosphates, if needed. Terminal phosphate removal is preferably done with a heat-inactivated phosphatase. Phosphatase activity is preferably removed prior to RE digestion and adapter ligation step in order to prevent interference with the intended ligation of adapters to doubly cut fragments. Heat inactivation allows phosphatase removal without a separation or extraction step. A preferred phosphatase comes from cold living Barents Sea (arctic) shrimp (U.S. Biochemical Corp.) ("shrimp alkaline phosphatase" or "SAP"). Terminal phosphate removal need be done only once for each population of cDNA being analyzed. In other embodiments alternative phosphatases can be used for terminal phosphate removal, such as calf intestinal phosphatase-alkaline from Boehringer Mannheim (Indianapolis, IN). Those that are not heat inactivated require a step to

separate the phosphatase from the cDNA sample before the RE/ligase reactions, such as by phenol-chloroform extraction.

The prepared cDNA is then separated into batches of from 1 picogram ("pg") to 200 nanograms ("ng") of cDNA each, 5 and each batch is separately processed by the further steps of the method. A number of batches sufficient for whichever QEA™ mode is to be practiced are made. For a tissue mode experiment, to analyze gene expression, preferably, from a majority of expressed genes in a human tissue, the presence 10 of about 15,000 distinct cDNA sequences needs to be determined. By way of example, one sample is divided into approximately 50 batches, each batch is then subject to an RE/ligase recognition reaction to generate approximately 200-500 fragments, and more preferably 250 to 350 fragments of 10 15 to 1000 bp in length, the majority of fragments preferably having a distinct length and being uniquely derived from one cDNA sequence. A preferable tissue mode analysis entails approximately 50 batches generating approximately 300 bands each. For query mode experiments, fewer recognition 20 reactions are employed since only a subset of the expressed genes are of interest, perhaps approximately from 1 to 100. The number of recognition reactions in an experiment can then number approximately from 1 to 10 and an approximately from 1 to 10 cDNA batches are prepared.

25 Following cDNA preparation is the important step of simultaneous RE cutting of and adapter ligation to the sample cDNA sequences. The prepared sample is cut with one or more REs. The number of REs and associated adapters preferably are limited so that both a compressed length distribution 30 consisting of shorter fragments is avoided and enough distinguishable labels are available for all the REs used. Alternatively, REs can be used without associated adapters in order that the amplified fragments not have the associated 35 recognition sequences. Absence of these sequences can be used to additionally differentiate genes that happen to produce fragments of identical length with particular REs.

In the same reaction mix, herein called the Qlig mix, REs, adapters and ligase enzyme are simultaneously present for concurrent adapter ligation and RE cutting. The amount of RE enzyme in the reaction is preferably approximately a 10 fold unit excess. Substantially greater quantities are less preferred because they can lead to star activity (non-specific cutting), while substantially lower quantities are less preferred because they will result in less rapid and only partial digestion and hence incomplete and inaccurate characterization of the subsequence distribution. REs and corresponding adapters are chosen according to the previous description. Table 10 in Sec. 6.10 lists exemplary REs and corresponding primers and linkers. Table 11 in Sec. 6.10.1 lists exemplary combinations for biotin labeled primers. The method is adaptable to any ligase enzyme that is active in the temperature range 10 to 37°C. T4 DNA ligase is the preferred ligase. In other embodiments, cloned T4 DNA ligase or T4 RNA ligase can also be used. In a further embodiment, thermostable ligases can be used, such as Ampligase™ Thermostable DNA Ligase from Epicentre (Madison, WI), which has a low blunt end ligation activity. These ligases in conjunction with the repetitive cycling of the basic thermal profile for the RE-ligase reaction, described in the following, permit more complete RE cutting and adapter ligation.

Also present in the Qlig mix are necessary buffers, as known in the art, and ATP. An excess of primers is preferably present in the Qlig mix in order than subsequent amplification can be performed in an automated manner. Preferably primers and linkers are present approximately in the ratio of 20:1 and to an adequate total primer amount of approximately 20 pm where 1 ng of cDNA is used. Less preferably the ratio is 10:1. Also, Betaine (Sigma Chemicals) is preferably present in the Qlig reaction mix. Betaine has been found to improve the uniformity of signals from fragments that are at approximately the same original

concentration by aiding ligation activity. Betaine also improves the PCR amplification of hard to amplify products.

RE/ligase reaction conditions are optimized to minimize unwanted products. As previously explained, 5 terminal phosphate removal from cDNA samples prevents unwanted ligation of cDNA blunt ends together and subsequent exponential amplification of the resulting dimers. Another class of unwanted products are fragment concatamers, formed when the sticky ends of cut cDNA fragments hybridize and 10 ligate together. Fragment concatamers are removed by maintaining restriction enzymes activity during ligation in order to cut any unwanted concatamers. Further, ligated primers terminate further RE cutting, since primers do not recreate RE recognition subsequences. A high molar excess of 15 adapters is, therefore, preferable to limit concatamer formation by driving the RE and ligase reactions toward complete digestion and adapter ligation. Finally, unwanted adapter self-ligation is prevented since primers and linkers lack terminal phosphates (preferably due to synthesis without 20 phosphates or less preferably due to pretreatment thereof with phosphatases).

The temperature profile of the RE/ligase reaction is important for complete cutting and ligation. The preferred protocol has several steps. The first step is at 25 the optimum RE temperature for a time sufficient to achieve substantially complete cutting, for example 37 °C for 30 minutes. The ligase used is preferably active during the first step. The second step is a ramp at -1 °C/min down to an optimum temperature for adapter annealing and primer 30 ligation, for example, 16 °C. The third step achieves substantially complete primer ligation of cut products, and is, for example, at 16 °C for 60 minutes. The REs used are preferably active during this third step. The fourth step is again at the temperature for optimum RE activity to achieve 35 complete cutting of recognition sites and unwanted ligation products, for example at 37 °C for 15 minutes. The fifth step is to heat inactivate the Qlig enzymes and is, for

example, above 65 °C. If the PCR amplification is to be performed immediately, as in the preferred single tube protocol of Sec. 6.4.1., this fifth step is at 72 °C for 20 minutes and performs additional reactions to be subsequently described. If the PCR amplification is not to be immediately performed, the Qlig reaction results are held at 4 °C, as in the much less preferred multi-tube protocol as Sec. 6.4.5. This temperature profile, together with the subsequent PCR profile, is illustrated in Fig. 16D.

10 A less preferred profile involves repetitive cycling of the first four steps of the temperature protocol described above, that is from an optimum RE temperature to an optimum annealing and ligation temperature, and back to an optimum RE temperature. The additional temperature cycles 15 act to further drive the RE/ligase reactions to completion. With this profile, it is preferred to use thermostable ligase enzymes. The majority of restriction enzymes are active at the conventional 16 °C ligation temperature and hence prevent unwanted ligations without thermal cycling. However, 20 temperature profiles comprising alternating optimum ligation conditions and optimum RE conditions can cause both enzymatic reactions to proceed more rapidly than if at one constant temperature. An exemplary profile comprises periodically cycling between a 37 °C optimum RE temperature to a 16 °C 25 optimum annealing and ligation temperature at a ramp of -1 °C/min, then to a 16 °C optimum ligation temperature, and then back to the 37 °C optimum RE temperature. Following completion of approximately 2 to 4 of these temperature cycles, the RE and ligase enzymes are heat inactivated by a 30 final stage above 65 °C for 10 minutes.

These thermal profiles are easily controlled and automated by the use of commercially available computer controlled thermocyclers, for example from MJ Research (Watertown, MA) or Perkin Elmer (Norwalk, CT).

35 The Qlig mix and reaction temperature profile are designed to achieve the substantially complete cutting of all RE recognition sites present in the analyzed sequence mixture

and the substantially complete ligation of primers to cut ends, each primer being unique in one reaction for one particular RE cut end. The fragments generated are limited by adjacent RE recognition sites, with substantially no 5 fragments having an internal undigested sites. Further, a minimum of unwanted self-ligation products and concatamers is formed. This invention is adaptable to other temperature profiles which achieve the same effect of substantially complete cutting and ligation. Exemplary alternative 10 profiles are described in the accompanying examples in Sec. 6.4.

Following the RE/ligase step is a step for amplifying the doubly cut cDNA fragments. Although PCR protocols are described in the exemplary embodiment of this 15 invention, any amplification method that selects fragments to be amplified based on end sequences is adaptable to this invention (see above). With high enough sensitivity of detection means, or even single molecule detection means, the amplification step can be dispensed with entirely. This is 20 preferable as molecular amplification often distorts the quantitative response of this method.

PCR amplification protocols used in this invention are designed to have maximum specificity and reproducibility. First, PCR amplification produces fewer unwanted products if 25 the linkers remain substantially melted and unable to initiate DNA strands, such as by performing all amplification steps at a temperature near or above the T_m of the linker. Second, amplification primers, typically strand 203 of Fig. 2A (and 304 of Fig. 3A), are preferably designed for high 30 amplification specificity by having a high T_m , preferably above 50 °C and most preferably above 68 °C, to ensure specific hybridization with a minimum of mismatches. They are further chosen not to hybridize with any native cDNA species to be analyzed. The previously described phasing 35 primers, which are alternatively used for PCR amplification, have similar properties. Third, the PCR temperature profile is preferably designed for specificity and reproducibility.

High annealing temperatures minimize primer mis-hybridizations. Longer extension times reduce PCR bias related to smaller fragments. Longer melting times reduces PCR amplification bias related to high G+C content. A 5 preferred PCR temperature cycles is 95 °C for 30 sec., then 57 °C for 1 min., then 72 °C for 2 min. This preferred PCR temperature profile is illustrated in Fig. 16D. Fourth, it is preferable to include Betaine in the PCR reaction mix, as this has been found to improve amplification of hard to 10 amplify products. To further reduce bias, large amplification volumes and a minimum number of amplification cycles, typically between 10 and 30 cycles, are preferred.

Any other techniques designed to raise specificity, yield, or reproducibility of amplification are applicable to 15 this method. For example, one such technique is the use of 7-deaza-2'-dGTP in the PCR reaction in place of dGTP. This has been shown to increase PCR efficiency for G+C rich targets (Mutter et al., 1995, *Nuc. Acid Res.* 23:1411-1418). For a further example, another such technique is the addition 20 of tetramethylammonium chloride to the reaction mixture, which has the effect of raising the T_m (Chevet et al., 1995, *Nucleic Acids Research* 23(16):3343-3344).

It can be advantageous to process multiple identical samples of RE/ligase reaction products, e.g. the 25 processed Qlig mix, with multiple PCR amplifications. Amplifications of multiple identical samples with the same number of cycles serves to check reliability and quantitative response by comparing signals from each of the separately amplified aliquots. Amplifications of multiple identical 30 samples with an increasing number of amplification cycles, for example 10, 15, and 20 cycles, are preferable in that amplifications with a lower number of cycles can detect more prevalent fragments in a more quantitative manner, while amplification with a higher number of cycles can detect less 35 prevalent fragments but less quantitatively.

It is preferable to process PCR amplification in the same reaction tube as the RE/ligase reaction, as this

promotes automation. First, a PCR reaction mix, herein called the QPCR mix, is made from appropriate DNA polymerases, dNTPs, and PCR buffer, but without any primer strands. Exemplary QPCR mix compositions can be found in the 5 examples of Sec. 6.4. The QPCR mix is placed in a reaction tube, and a layer of wax melting near but below 72 °C is layered above the QPCR mix. The Qlig mix is placed above the wax layer and processed according to the previously described temperature profile, which does not melt the wax. When the 10 RE/ligase reactions are complete, the tube is incubated at 72°C for 20 min. This incubation melts the linkers from the fragments, melts the wax layer and allows the processed Qlig mix and the QPCR mix to combine, and finally, permits the DNA polymerase to complete the fragments to blunt-ended dsDNA.

15 After this incubation, the PCR temperature profile is performed according to the preferred protocol for a certain number of cycles.

It is important in the preferred single tube embodiment that the Qlig and QPCR mixes do not intermingle 20 before the intended step. Even slight mixing due to hairline cracks in the wax layer can contaminate the reactions. The preferred wax to prevent such intermingling is a mixture of Paraffin wax and Chillout™ 14 wax in a 90:10 ratio, respectively. The paraffin is a highly purified paraffin wax 25 melting between 58 °C and 60 °C such as can be obtained from Fluka Chemical, Inc. (Ronkonkoma, N.Y.) as Paraffin Wax cat. no. 76243. Chillout 14 Liquid Wax is a low melting, purified paraffin oil available from MJ Research. This wax layer is created in the following manner. The reaction tubes are pre- 30 waxed by melting the preferred wax onto the upper half of the sides of the tubes. The QPCR mix is added carefully avoiding this wax layer. Then the wax layer is melted onto the surface of the QPCR mix by incubating the tubes at 75°C for 2 min. The wax layer is then carefully solidified by 35 decreasing the temperature of the tubes by 5°C every 2 min. until a final temperature of 25°C is reached. The Qlig mix is then gently added on top of this wax surface. This single

tube protocol is adaptable to other less preferable waxes that melt at approximately at 72°C, such as Ampliwax beads (Perkin-Elmer, Norwalk, CT). Further, other so called PCR "hot-start" procedures can be used, such as those employing 5 heat sensitive antibodies (Invitrogen, CA) to initially block the activity of the polymerase.

Alternatively, PCR amplification can be performed in a separate tube. In this case the QPCR mix is prepared in a second tube. The first tube with the processed Qlig mix is 10 incubated at 72°C for approximately 10 min. in order to melt the linker from the fragments. An aliquot of the Qlig mix is then combined with the QPCR mix in the second tube, and a further incubation at 72°C for 10 minutes completes the fragments to blunt-ended dsDNA. After this incubation, the 15 PCR temperature profile is performed according to the preferred protocol for a certain number of cycles.

Following the amplification step, optional cleanup and separation steps prior to length separation and fragment detection can be advantageous to substantially eliminate 20 certain unwanted DNA strands and thereby to improve the signal to noise ratio of QEA™ signals, or to substantially separate the reaction products into various classes and thereby to simplify interpretation of detected fragment patterns by removing signal ambiguities. For example, unused 25 primer strands and single strands produced by linear amplification are unwanted in later steps. These steps are based on previously described primer enhancements including conjugated capture moieties and release means.

In one embodiment of these optional steps where one 30 of the two primers used has a conjugated capture moiety, QEA™ reaction products fall into certain categories. These categories, described without limitation in the case where the capture moiety is biotin, are:

- a) dsDNA fragments neither strand of which has a biotin 35 moiety;
- b) dsDNA fragments having only one strand with a conjugated biotin moiety;

- c) dsDNA molecule fragments having biotin moieties conjugated to both strands; and
- d) unwanted ssDNA strands with and without conjugated biotin.

5 The additional method steps comprise contacting the amplified fragments with streptavidin affixed to a solid support, preferably streptavidin magnetic beads, washing the beads to in a non-denaturing wash buffer to remove unbound DNA, and then resuspending the beads in a denaturing loading buffer 10 and separating the beads from this buffer. The denatured single strands are then passed to the separation and detection steps.

As a results of these steps only the strand of category "b" without biotin is removed in the loading buffer 15 for separation and detection. Thereby, only fragments cut on either end by different REs and freed from single stranded contaminants are separated and detected with minimized noise. Category "a" products are not bound to the beads and are washed away in the non-denaturing wash buffer. Similarly, 20 class "d" products without biotin moieties are washed away. All products with a conjugated biotin are retained by the streptavidin beads after washing. The denaturing loading buffer denatures categories "b" and "c" products attached to the beads, but both strands of category "c" products have 25 conjugated biotin and remain attached to the beads. Similarly, class "d" products with conjugated biotin are retained by the beads.

In another embodiment, the biotinylated primer can include a release means in order to recover fragments of 30 class "c". After the step of suspension in a denaturing buffer, the releasing means, e.g. UDG or AsCI, can be applied to release the biotinylated strands for separation and detection. Fragments detected at this second separation in addition to those previously detected then represent class 35 "c" products.

Further embodiments will be apparent to those of skill in the art. For example, two or more types of capture

moieties can be used in a single reaction to separate different classes of products. Capture moieties can be combined with release means to achieve similar separation. Label moieties can be combined with capture moieties to verify separations or to run reactions in parallel.

This invention is adapted to other less preferred means for single strand separation and product concentration that are known in the art. For example, single strands can be removed by the use of single strand specific exonucleases. 10 Mung Bean exonuclease, Exo I or S1 nuclease can be used, with Exo I preferred because of its higher specificity for single strands while S1 is least preferred. Other methods to remove unwanted strands include the affinity based methods of gel filtration and affinity column separation. Amplified 15 products can be concentrated by ethanol precipitation or column separation.

The last QEA™ step is separation according to length of the amplified fragments followed by detection the fragment lengths and end labels (if any). Lengths of the 20 fragments cut from a cDNA sample typically span a range from a few tens of bp to perhaps 1000 bp. Any separation method with adequate length resolution, preferably at least to three base pairs in a 1000 base pair sequence, can be used. It is preferred to use gel electrophoresis in any adequate 25 configuration known in the art.

Gel electrophoresis is capable of resolving separate fragments which differ by three or more base pairs and, with knowledge of average fragment composition and with correction of composition induced mobility differences, of 30 achieving a length precision down to 1 bp. A preferable electrophoresis apparatus is an ABI 377 (Applied Biosystems, Inc.) automated sequencer using the Gene Scan software (ABI) for analysis. The electrophoresis can be done by suspending the reaction products in a loading buffer, which can be non-35 denaturing, in which the dsDNA remains hybridized and carries the labels (if any) of both primers. The buffer can also be denaturing, in which the dsDNA separates into single strands

that typically are expected to migrate together (in the absence of large average differences in strand composition or significant strand secondary structure). The length distribution is detected with various detection means. If no labels are used, means such as Ag staining and intercalating dyes can be used. Here, it can be advantageous to separate reaction products into classes, according to the previously described protocols, in order that each band can be unambiguously identified as to its target end subsequences.

10 In the case of fluorochrome labels, since multiple fluorochrome labels can be typically be resolved from a single band in a gel, the products of one recognition reaction with several REs or other recognition means or of several separate recognition reactions can be analyzed in a 15 single lane. However, where one band reveals signals from multiple fluorochrome labels, interpretation can be ambiguous: is such a band due to one fragment cut with multiple REs or to multiple fragments each cut by one RE. In this case, it can also be advantageous to separate reaction 20 products into classes.

Preferred protocols for the specific RE embodiments are described in detail in Sec. 6.4.

5.2.3. THE SEQ-QEA™ EMBODIMENT

25 SEQ-QEA™ is an alternative embodiment of the preferred method of practicing a RE/ligase embodiment of QEA™ method as previously described in Sec. 5.2.2. By the use of adapters comprising specially constructed primers bearing a recognition site for a Type IIS RE, a SEQ-QEA™ method is able 30 to identify an additional 4-6 terminal nucleotides adjacent to the recognition subsequence of the RE initially cutting a fragment. Thereby, the effective target subsequence is the concatenation of the initial RE recognition subsequence and the additional 4-6 terminal nucleotides, and has, therefore, 35 a length of at least from 8 to 12 nucleotides and preferably has a length of at least 10 nucleotides. This longer effective target subsequence is then used in QEA™ analysis

methods as described in Sec. 5.4 ("QEA™ Analysis and Design Methods") which involves searching a database of sequences to identify the sequence or gene from which the fragment derived. The longer effective target subsequence increases 5 the capability of these methods to determine a unique source sequence for a fragment.

In this section, for ease of description and not limitation, first shall be described Type IIS REs, next the specially constructed primers, and then the additional method 10 steps of a SEQ-QEA™ method used to recognize the additional nucleotides.

A Type IIS RE is a restriction endonuclease enzyme which cuts a dsDNA molecule at locations outside of the recognition sequence of the Type IIS RE (Szybalski et al., 15 1991, *Gene* 100:13-26). Fig. 17C illustrates Type IIS RE 1731 cutting dsDNA 1730 outside of its recognition subsequence 1720 at locations 1708 and 1709. The Type IIS RE preferably generates an overhang by cutting the two dsDNA strands at locations differently displaced away on the two strands from 20 the recognition sequence. Although the recognition subsequence and the displacement(s) to the cutting site(s) are determined by the RE and are known, the sequence of the generated overhang is determined by the dsDNA cut, in particular by its nucleotide sequence outside of the Type IIS 25 recognition region, and is, at first, unknown. Thus in a SEQ-QEA™ embodiment the overhangs generated by the Type IIS REs are sequenced. Table 17 in Sec. 6.10.1 lists several Type IIS REs adaptable for use in the SEQ-QEA™ method and their relevant characteristics, including their recognition 30 subsequences on both DNA strands and the displacements from these recognition subsequences to the respective cutting sites. It is preferable to use REs of high specificity and generating an overhang of at least 4 bp displaced at least 4 or 5 bp beyond the recognition subsequence in order to span 35 the remaining recognition subsequence of the RE that initially cut the fragment. *FokI* and *BbvI* are most preferred Type IIS REs for the SEQ-QEA™ method.

Next, the special primers, and the special linkers if needed, which hybridize to form the adapters for SEQ-QEA™, have, in addition to the structure previously described in Sec. 5.2.1, a Type IIS recognition subsequence whose placement is important in order that the overhang generated by the Type IIS enzyme be contiguous to the initial target end subsequence. The placement of this additional subsequence is described with reference to Figs. 17A-E, which illustrate steps in a SEQ-QEA™ alternative embodiment. Fig. 17B schematically illustrates dsDNA 1702, which is a fragment cut from an original sample sequence on one end by a first RE and on the other end by a different second initial RE, with adapters fully hybridized but prior to primer ligation. Thus, linker strand 1711 has hybridized to primer strand 1712 and to the 5' overhang generated by the first RE, and how fixes primer 1712 adjacent to fragment 1702 for subsequent ligation. Primer 1712 has recognition subsequence 1720 for Type IIS RE 1721. Linker 1711, to the extent it overlaps and hybridizes with recognition subsequence 1720, has complementary recognition subsequence 1721. Additionally, primer 1712 preferably has a conjugated label moiety 1734, e.g. a fluorescent FAM moiety. Similarly, linker strand 1713 has hybridized to primer strand 1714 and to the 5' overhang generated by the second RE. Primer 1714 preferably has a conjugated capture moiety 1732, e.g. a biotin moiety, and a release means represented by subsequence 1723.

Subsequence 1704 terminating at nucleotide 1707 in Fig. 17B is the portion of the recognition subsequence of the first RE remaining after its cutting of the original sample sequence. The placement of the Type IIS RE recognition subsequence is determined by the length of this subsequence. Fig. 17A schematically illustrates how the length of subsequence 1704 is determined by properties of the first RE. The first initial RE is chosen to be of a type that recognizes subsequence 1703, terminating with nucleotide 1707, of sample dsDNA 1701, and that cuts the two strands of dsDNA 1701 at locations 1705 that are located within

recognition subsequence 1703. In order that the first RE recognize a known target subsequence, it is highly preferable that subsequence 1703 be entirely determined by the first RE and be without indeterminate nucleotides. As a result of 5 this cutting, overhang subsequence 1706 is generated and has a known sequence, since it is entirely within the determined recognition subsequence 1703. Thereby, subsequence 1704, the portion of the recognition subsequence 1703 remaining on a fragment cut by the first RE, has a length not less than the 10 length of overhang 1706 and is typically longer. Typically and preferably, subsequence 1703 is of length 6 and is palindromic; locations 1705 are symmetrically placed in subsequence 1703; and overhang 1706 is of length 4. Therefore, the typical length of the remaining portion 1704 15 of the recognition subsequence 1703 is of length 5.

The preferred placement of Type IIS recognition sequence 1720 is now be described with reference to Fig. 17C, which schematically illustrates dsDNA 1730, which derives from dsDNA 1702 of Fig. 17B after the further steps of primer 20 ligation, PCR amplification with primers 1712 and 1714, binding of capture moiety 1732 to binding partner 1733 affixed to a solid-phase substrate, and binding of Type IIS RE 1731 to its recognition subsequence 1720. Subsequence 1722 is the subsequence between recognition subsequence 1720 25 and the end of primer 1712 at location 1705. Type IIS RE is illustrated cutting dsDNA 1730 at nucleotide locations 1708 and 1709 and, thereby, generating an exemplary 5' overhang 1724 between these locations. For this overhang to be contiguous with the remaining portion 1704 of initial target 30 end subsequence 1703, nucleotide 1709 is adjacent to nucleotide 1707 terminating subsequence 1704. Therefore, Type IIS recognition sequence 1720 is preferably placed on primer 1712 such that the length of subsequence 1704 plus the length of subsequence 1722 equals the distance of closest 35 cutting of Type IIS RE 1731. For example, in the case of FokI, since the closest cutting distance is 9 and the typical length of subsequence 1704 is 5, its recognition sequence is

preferably placed 5 bp from the end of primer 1712. In the case of BbvI, since the closest cutting distance is 8, its recognition sequence is preferably placed 3 bp from the end of primer 1712.

5 Finally, Fig. 17D schematically illustrates dsDNA 1730 after cutting by Type IIS RE 1731. dsDNA has 5' overhang 1724 between and including nucleotides 1708 and 1709, where the Type IIS RE cut dsDNA 1730 of Fig. 17C. This overhang is contiguous with former subsequence 1704, the 10 remaining portion of the recognition sequence of the first RE, which has been cut off. The shorter strand has primer 1714 including release means represented by subsequence 1723. dsDNA 1730 remains bound to the solid-phase support through capture moiety 1732 and binding partner 1724. The absence of 15 label moiety 1734 can be used to monitor the completeness of cutting by Type IIS RE 1731.

This invention is also adaptable to other less preferable placements of recognition sequence 1720. If recognition sequence 1720 is placed closer to the 3' end of 20 primer 1712 than the optimal and preferable distance, the overhang produced by Type IIS RE 1731 is not contiguous with recognition subsequence 1703 of the first RE, and a contiguous effective target subsequence is not generated. In this case, optionally, the determined sequence of the Type 25 IIS RE generated overhang can be used as third internal subsequence information in QEA™ experimental analysis methods in order to further resolve the source sequence of fragment 1702, if necessary. If recognition sequence 1720 is placed further from the 3' end of the cut primer than the optimal 30 and preferable distance, the overhang produced by Type IIS RE overlaps with recognition subsequence 1703 of the first RE. In this case, the length of the now contiguous effective target subsequence is less than the sum of the lengths of the Type IIS overhang and the first RE recognition subsequence. 35 Effective target end subsequence information is, thereby, lost. In case recognition sequence 1710 is placed further

from the 3' end than the distance of furthest cutting, no additional information is obtained.

Primer 1714 also has certain additional structure. First, primer 1714 has capture moiety 1732 conjugated near or 5 to its 5' end. Biotin/streptavidin are the preferred capture moiety/binding partner pair, which are used in the following description without limitation to this invention. Second, primer 1714 has release means represented as subsequence 1723. As previously described, the release means allows 10 controlled release of strand 1735 of Fig. 17D from the capture moiety/binding partner complex. This alternative is adaptable to any such controlled release means, including the cases where subsequence 1723 is one or more uracil 15 nucleotides and where it is the recognition subsequence of an RE which cuts extremely rarely if at all in the sequences of the sample, e.g. AscI. Release means are particularly useful in the case of biotin-streptavidin, which form a complex that is difficult to dissociate.

Table 18 of Sec. 6.10.1 lists exemplary primers, 20 linkers, and associated REs, for the preferred implementation of SEQ-QEA™ in which contiguous effective target end subsequences are formed. This description has illustrated the generation of a 5' Type IIS generated overhang. Primers can equally be constructed to generate a less preferable 3' 25 overhang by using a Type IIS whose closest cutting distance is on the 3' strand, rather than on the 5' strand.

Finally, the method steps of SEQ-QEA™ are now described. SEQ-QEA™ comprises, first, practicing the RE/ligase embodiment of QEA™ using the special primers and 30 linkers previously described followed, second, by certain additional steps unique to SEQ-QEA™. Figs. 17B-E illustrate various steps in a SEQ-QEA™ method. Fig. 17B illustrates a fragment from a sample sequence digested by two different REs and just prior to primer ligation. Fig. 17C illustrates a 35 sample sequence after primer ligation, chain blunt-ending, and PCR amplification. These QEA™ steps are preferably performed according to the embodiments described in Sec.

5.2.2, but can alternatively be performed by any RE/ligase embodiment. The additional steps unique to SEQ-QEA™ include, first, binding the amplified fragments to a solid-phase support, also illustrated in Fig. 17C, second, washing the 5 bound fragments, and third, digesting the bound fragments by the Type IIS RE corresponding to primer 1712 used. The Type IIS digestion is preferably performed with reaction conditions suitable to achieve complete digestion, which can be checked by insuring the absence of optional label moiety 10 1734 after washing the bound, digested sequences. Fig. 17D illustrates dsDNA fragments 1730 remaining after complete digestion by the Type IIS RE. Before Type IIS digestion, an aliquot of the bound, amplified RE/ligase reaction products is denatured and the supernatant, containing the labeled 5' 15 strands, are separated according to length by, e.g., gel electrophoresis, in order to determine the length of each fragment doubly cut by different REs.

The subsequent additional SEQ-QEA™ step is sequencing of overhang 1724. This can be done in any manner 20 known in the art. In a preferred embodiment suitable for lower fragment quantities, an alternative, herein called a phasing QEA™ method, can be used to sequence this overhang. Phasing QEA™ depends on the precise sequence specificity with 25 which RE/ligase reactions recognize short overhangs, in this case the Type IIS generated overhang. Fig. 17E illustrates a first step of this embodiment in which a QEA™ method adapter, which is comprised of primer 1751 with label moiety 1753 and linker 1750, has hybridized to overhang 1724 in Type IIS digested fragment 1730 bound to a solid-phase support. By 30 way of example only, overhang 1724 is here illustrated as being 4 bp long. In this embodiment, special phasing linkers are used. For each nucleotide position of overhang 1724, e.g. position 1754, 4 pools of linkers 1750 are prepared. All linkers in each pool have one fixed nucleotide, i.e. one 35 of either A, T, C, or G, at that position, e.g. position 1755, while random nucleotides in all combinations are present at the other three positions. For each nucleotide

position of the overhang, four RE/ligase reactions are performed according to QEA™ protocols, one reaction using linkers from one of the four corresponding pools. Linkers from only one pool, that having a nucleotide complementary to 5' overhang 1724 at position 1754, hybridize without error, and only these linkers can cause ligation of primer 1751 to the 5' strand of fragment 1730. When the results of the four RE/ligase reactions are denatured and separated according to length, only one reaction of the four can produce labeled 10 products at a length corresponding to the length of fragment 1730, namely the reaction with linkers complementary to position 1754 of overhang 1724. Thereby, by performing four RE/ligase reactions for each nucleotide position of overhang 1724, this overhang can be sequenced. Optionally, the 15 products of these four RE/ligase reactions can be further PCR amplified. In a further option, if linkers 1750 comprise subsequence 1756 that is uniquely related to the fixed nucleotide in subsequence 1752 and if four separately and distinguishably labeled primers 1751 complementary to these 20 unique subsequences are used, all four RE/ligase reactions for one overhang position can be simultaneously performed in one reaction tube. With this overhang sequencing alternative embodiment, release means 1723 can be omitted from primer 1714.

25 In an alternate embodiment, sequencing of a 5' overhang can be done by standard Sanger reactions. Thus strand 1735 is elongated by a DNA polymerase in the presence of labeled ddNTPs at a relatively high concentration to dNTPs in order to achieve frequent incorporation in the short 4-6 30 bp elongation. Partially elongated strands 1735 are released by denaturing fragment 1730, washing, and then by causing release means 1723 to release strands 1735 from the capture moiety bound to the solid phase support. The released, partially elongated strands are then separated by length, 35 e.g., by gel electrophoresis, and the chain terminating ddNTP is observed at the length previously observed for that

fragment. In this manner, the 4-6 bp overhang 1724 of each fragment can be quickly sequenced.

The effective target subsequence information, formed by concatenating the sequence of the Type IIS overhang 5 to the sequence of the recognition subsequence of the first RE, is then input into QEA™ Experimental Analysis methods, and is used as a longer target subsequence in order to determined the source of the fragment in question. This longer effective target subsequence information preferably 10 permits exact and unique sample sequence identification.

5.2.4. 5'-QEA™ ALTERNATIVE RE EMBODIMENT

In QEA™ embodiments of this invention, it is important that the one or more fragments of a nucleic acid 15 from a sample which are generated by the recognition reactions be of definite length, that is that the length of each fragment depends only on the sequence of the nucleic acid and not on experimental conditions, e.g., the synthesis conditions of the nucleic acid. Further, it is important for 20 the experimental analysis and design methods of Sec. 5.4 that the length of a fragment be precisely predictable from the nucleotide sequence of the sample nucleic acid. In the preferred RE/ligase embodiments of QEA™, these goals are accomplished primarily by selecting signals from fragments 25 doubly cut on both ends by one or more REs. The nucleotide distance between adjacent RE recognition subsequences is determined only by the sequence of nucleic acid from the sample. Also the described alternatives and extensions generate additional signal information dependent only on the 30 nucleic acid sequence. In these embodiments, nucleic acid, e.g. cDNA, synthesis conditions are then only of indirect importance, in that they preferably adequately represent input mRNA.

Other RE/ligase embodiments utilize signals from 35 fragments of a nucleic acid that, although only singly cut by an RE on one end, nevertheless have a definite length, dependent only on nucleotide sequence, because of particular

cDNA synthesis conditions that fix the other end. For these embodiments, therefore, the cDNA synthesis conditions are of direct importance, in that these embodiments can only be used with cDNA synthesized according to the particular conditions.

5 In general, these conditions insure that the cDNA begins or ends in a known relation, herein called "anchored," to general landmarks on the input mRNA. In particular, preferable anchoring landmarks include the 5' end of the poly(A)⁺ tail present on the 3' end of the input mRNA, or the 10 cap on the 5' end of the input mRNA. For example, cDNA fragments terminated on their 5' end in a fixed relation to the 5' cap of the source mRNA and cut on their 3' end at the nearest recognition subsequence of a single RE have a definite length and generate QEA™ signals that can be used to 15 determine the source nucleic acid in the sample. Similarly, cDNA fragments terminated on their 3' end in a fixed relation to the 5' end of the poly(A)⁺ tail present on the source mRNA and cut on their 5' end at the nearest recognition sequence of a single RE also have a definite length and generate QEA™ 20 signals that can also be used to determine the source nucleic acid in the sample.

Turning first the case of 5' anchored cDNA, such cDNA can be synthesized by a protocol which requires the presence of an intact 5' cap on the input mRNA. One such 25 exemplary preferred protocol is described in Sec. 6.3.3. This protocol depends upon using a RNA ligase to ligate to a source mRNA at the nucleotide adjacent to the 5' cap a DNA-RNA chimera comprising a first DNA subsequence 5' to the ribonucleotide triplet GGA at the 3' end of the chimera. The 30 RNA component of the DNA-RNA chimera is preferably GGA, but any RNA subsequence can be used that promotes effective ligation by the ligase chosen of the chimera to the source mRNA. The DNA oligonucleotide component is later used as a primer and is herein called a "5'-cap-primer" 35 oligonucleotide. This ligation is accomplished by dephosphorylating input mRNA with an alkaline phosphatase and then cleaving the 5' cap with an acid pyrophosphatase,

preferably tobacco acid pyrophosphatase, leaving a 5' phosphate needed for ligation only on mRNAs having a 5' cap. During the ligation step, an excess of primer is used to prevent self-ligations of the input mRNA. The preferred RNA 5 ligase is T4 RNA ligase. First strand synthesis is then performed with a first DNA primer comprising the first DNA subsequence. Thereby, all cDNAs originate from input mRNAs having their 5' cap. Second strand synthesis is then performed with such second strand primers as are known in the 10 art. Preferably second strand primers are three second strand primers mixed or in separate pools, each of which comprises a second DNA subsequence 5' to one of three oligo(dT) one-nucleotide phasing primers, as known in the art (Liang et al., 1994, *Nuc. Acid Res.* 22:5763-5764). 15 Alternatively, other primers known in the art could be used, including, a single oligo(dT) primer, a sequence specific primer, or random primers. For small amounts of input mRNA, the first DNA primer and a second DNA primer comprising the second DNA subsequence can be used in a PCR reaction to 20 amplify the synthesized cDNA. This QEA™ embodiment is adaptable to other methods known in the art to produce cDNAs with a 5' end anchored in a fixed relation to the 5' mRNA cap, for example the CapFinder™ PCR cDNA Library Construction Kit Clonetech (Palo Alto, CA). See also Schmidt et al., 25 1996, *Nuc. Acids. Res.* 24:1789-1791.

The first and second DNA primer sequences are preferably chosen according to certain guidelines. First, they are chosen not to generate by themselves any PCR products from the cDNA sample nucleic acids. Second, they 30 are of a sufficient length and average base content (approximately 60% G+C) to hybridize in high stringency conditions. Third, they have no significant secondary structure. Finally, they can have included RE recognition sites, initiators, etc. to promote later cloning or 35 expression. Exemplary first and second primers are described in Sec. 6.3.3. Software packages are available for primer construction according to such guidelines, an example being

OLIGO™ Version 4.0 For Macintosh from National Biosciences, Inc. (Plymouth, MN).

Having cDNA synthesized according to the exemplary 5' anchoring protocol, the 5'-QEA™ embodiment is performed 5 according to the general methods Sec. 5.2.2, including the optional cleanup and separation steps. In particular, the QPCR mix is prepared as previously described. The Qlig mix includes the one RE chosen to cut the fragment and an associated adapter with primer excess. These primers are 10 preferably be labeled are most preferably do not have a conjugated capture moiety. Also included in the Qlig mix in a quantity sufficient for PCR amplification is an extra primer, which is the first DNA primer, that is the DNA portion of the chimera now appearing on the 5' end of the 15 synthesized cDNA, together with a conjugated biotin moiety or other capture moiety,. The RE/ligase reactions and the subsequent PCR amplification are performed as previously described and result in the following classes of fragments. First, there are fragments singly cut by the chosen RE which 20 are exponentially amplified because of the presence of the first DNA primer and which have on their 5' ends the biotin labeled first DNA primer. Second, there are exponentially amplified fragments doubly cut by the chosen RE which have no biotin labels. Third, there can be linearly amplified, non- 25 labeled, singly cut fragments. After contacting these reaction products with streptavidin beads and washing, only the first class of fragments is retained, that is fragments singly cut adjacent to the 5' end. Upon resuspending the beads in a denaturing loading buffer, only the denatured 30 single strands from such fragments generate signals after the separation and detection steps. These signals have a definite length, because the RE recognition site nearest the 5' end is determined only by the sequence of the nucleic acid.

35 Turning to the less preferred case of 3' anchored cDNA, such cDNA can be synthesized by protocols known in the art which utilize phasing primers. Such phasing primers can

comprise a first DNA subsequence, which is constructed according to the previously described primer guidelines, 5' to one of three oligo(dT) one nucleotide phasing primer subsequences (Liang et al. 1994). Sequences MBTA, MBTC, and 5 MBTG of Sec. 6.3.3 are exemplary of such primers. The RE/ligase and PCR amplification reactions are carried out according to the protocol of the 5'-QEA™ embodiment with the exception that the extra primer used in the Qlig mix is the first DNA subsequence used in the prior cDNA synthesis with a 10 conjugated biotin or other capture moiety. After completion of the protocol, signals are only generated from fragments cut by the chosen RE adjacent to the 3' end. These signals have a definite length, because the RE recognition site nearest the 3' end is determined only by the sequence of the 15 nucleic acid.

The signals generated from the singly cut fragments according to the protocols of this section can be used in the computer implemented experimental analysis methods of Sec. 5.4 in order to determine the sample nucleic source of a 20 particular signal. The analysis methods need minimal adaptation in a manner that will be apparent to one of skill in the computer arts in order that the 5' or 3' end cDNA sequence is one of the target end sequences. This adaptation can be done in several ways, including simply specially 25 marking in the signals that one target end subsequence is the 3' or 5' end as needed or by including in the generated signal an artificial and not naturally occurring target subsequence that represents the 3' or the 5' end as appropriate and concatenating these artificial subsequences 30 to nucleic acid sequences input from a database prior to computer processing. Similar minimal adaptations to the computer implemented experimental design methods can be made in order to create and optimize experiments generating singly cut fragments.

35 The embodiments described in this section, in particular 5'-QEA™, can be practiced in combination with QEA™ embodiments herein described. It will be apparent to one of

skill in the art how such combinations can be performed. Specifically, it is advantageous to combine 5'-QEA™ with SEQ-QEA™ to obtain signals which include longer effective target subsequence information on the singly cut end along with 5 information on the distance of the effective target subsequence from the end of the cDNA.

5.2.5. FURTHER ALTERNATIVE RE EMBODIMENTS

The embodiments of this section remove unwanted RE/ligase reaction products at least partially by utilizing cDNA with conjugated capture moieties, obtained perhaps from either first and second strand synthesis with primers having conjugated capture moieties or from PCR amplification of cDNA with such primers. The preferred capture moiety is biotin 15 for which the corresponding binding partner is streptavidin attached to a solid support, preferably magnetic beads. These embodiments are adaptable to other capture moieties and corresponding binding partners.

A first QEA™ embodiment in conjunction with 20 sufficiently sensitive detection means can advantageously minimize or eliminate altogether the PCR amplification step. PCR amplification disadvantageously has a non-linear response well known in the arts, depending on such factors as fragment length, average base composition, and secondary structure. 25 To improve quantitative response, it is preferred to eliminate the PCR amplification step or at least to minimize the number of PCR cycles. Then output signal intensity is more nearly linearly responsive to the abundance of the input nucleic acids generating that signal.

30 In the previously described RE/ligase embodiments the amplification step serves both to amplify the signals from fragments of interest and simultaneously to dilute the signals from unwanted fragments without a definite sequence-dependent length and. For example, in the protocol of Sec. 35 5.2.2, fragments doubly cut with REs and ligated to adapters are exponentially amplified, while unwanted fragments singly cut by an RE are at best linearly amplified. After ten

cycles of amplification, since doubly cut fragments are amplified 1000X while singly cut fragments are amplified 10X, fragments from sample nucleic acids with a relative abundance of 1% or more can be detected above the background noise 5 while fragments from sample nucleic acids with a relative abundance of 1% or less can be lost in the unwanted background. More amplification cycles permit both greater sensitivity and greater ability to observe rare fragments from rare sequences.

10 More sensitive detection means decrease the need for amplification in order to generate observable signals. In the case of standard fluorescent detection means, a minimum of 6×10^{-12} moles of fluorochrome (approximately 10^5 molecules) is required for detection. Since one gram of cDNA 15 contains about 10^6 moles of transcripts, it is possible to detect transcripts to at least a 1% relative level from microgram quantities of mRNA. With greater mRNA quantities, proportionately rarer transcripts are detectable. Labeling and detection schemes of increased sensitivity permit use of 20 less mRNA. Such a scheme of increased sensitivity is described in Ju et al., 1995, Fluorescent energy transfer dye-labeled primers for DNA sequencing and analysis, Proc. Natl. Acad. Sci. USA 92:4347-4351. Possible single molecule detection means are about 10^5 times more sensitive than 25 existing fluorescent means (Eigen et al., 1994, Proc. Natl. Acad. Sci. USA 91:5740-5747).

To minimize or eliminate amplification steps, the first embodiment described in this section minimizes the need for amplification in order to dilute unwanted signals by 30 using a capture moiety to remove unwanted singly cut fragments from the doubly cut fragments of interest. In the protocols of Sec. 5.2.2, only the doubly cut fragments have definite lengths dependent only on the sequences of the input nucleic acids. Singly cut fragments have non-diagnostic 35 lengths depending also on cDNA synthesis conditions. In this protocol, PCR amplification can be optionally employed to generate sufficient signal intensity for detection. It is

not needed to minimize the background noise generated in the previously described protocols. The steps of this protocol comprise synthesis of cDNA using a primer labeled with a capture moiety, circularization of the cDNA, cutting with 5 REs, and ligation to adapters. Singly cut ends are then removed by contacting the reaction products with a solid phase to which the binding partner of the capture moiety is affixed.

Figs 4A, 4B, and 4C illustrate this alternative 10 protocol, which preferably uses biotin as a capture moiety for direct removal of the singly cut 3' and 5' cDNA ends from the RE/ligase reaction products. cDNA first strands are synthesized according to the method of Sec. 6.3.3 using, for example, an oligo(dT) primer with a biotin molecule linked to 15 a thymidine nucleotide. For example, such a primer is $T_nT(\text{biotin})T_m$, with n approximately equal to m , and with $n + m$ sufficiently large, approximately 12 to 20, so that the primer will reliably hybridize to the poly(A) tail of mRNA. Other biotin labeled primers may also be used, such as random 20 hexamers. Double stranded cDNA is then synthesized, also according to Sec. 6.3.3. In this embodiment, terminal phosphates are retained. Fig. 4A illustrates such a cDNA 401 with ends 407 and 408, poly(dA) subsequence 402, oligo(dT) primer 403 with biotin 404 attached. Subsequence 405 is the 25 recognition sequence for RE₁; subsequence 406 is the recognition sequence for RE₂. Fragment 409 is the cDNA sequence defined by these adjacent RE recognition sequences. Fragments 423 and 424 are singly cut fragments resulting from RE cleavages at subsequences 405 and 406.

30 Next, the cDNA is ligated into a circle. A ligation reaction using, for example, T4 DNA ligase is performed under sufficiently dilute conditions so that predominantly intramolecular ligations occur circularizing the cDNA, with only a minimum of intermolecular, concatamer 35 forming ligations. Reaction conditions favoring circularization versus concatamer formation are described in Maniatis, 1982, Molecular Cloning A Laboratory Manual, pp.

124-125, 286-288, Cold Spring Harbor, NY. A DNA concentration of less than approximately 1 μ g/ml has been found adequate to favor circularization. Concatamers can be separated from circularized single molecules by size 5 separation using gel electrophoresis, if necessary. Fig. 4B illustrates the circularized cDNA. Blunt end ligation occurred between ends 407 and 408.

Then the circularized, biotin labeled, cDNA is cut with REs and ligated to adapters uniquely recognizing and 10 perhaps uniquely labeled for each particular RE cut. The RE/ligase step is performed by procedures described in the sections hereinabove, for example in Sec. 5.2.2, so that RE digestion and primer ligation proceed to completion with minimal formation of concatamers and other unwanted ligation 15 products. Next, unwanted singly cut ends are removed by contacting the reaction products with streptavidin or avidin magnetic beads, leaving only doubly cut fragments that have RE-specific recognition sequences ligated to each end. Fig. 4C illustrates these steps. Sequences 405 and 406 are cut by 20 RE₁ and RE₂, respectively, and adapters 421 and 422 specific for cuts by RE₁ and RE₂, respectively are ligated onto the overhangs. Thereby, fragment 409 is freed from the circularized cDNA and adapters 421 and 422 are ligated to it. The remaining segment of the circularized cDNA comprises 25 singly cut ends 423 and 424 with ligated adapters 421 and 422. Both singly cut ends are joined to the primer sequence 403 with attached biotin 404. Removal is accomplished by contact with streptavidin or avidin 420 which is fixed to substrate 425, perhaps comprising magnetic beads. Doubly cut 30 labeled fragment 409, now separated from the singly cut ends, can be separated according to length and detected with minimized background noise signals.

Thereby, signals from the labeled doubly cut ends of interest can be directly detected with minimal 35 contamination from signals from unwanted labeled singly cut ends. Importantly, the detected signals more quantitatively reflect the relative abundance of the source cDNA, and thus

gene expression levels. Optionally, if the signal levels are too low for direct detection, the reaction products can be subjected to just the minimum number of cycles, for example according to the methods of Sec. 5.2.2, to detect the gene or sequence of interest. For example, the number of cycles can be as small as four to eight without any concern of background contamination or noise. Thus, in this embodiment, amplification is not needed to suppress signals from singly cut ends, and preferred more quantitative response signal intensities result.

Another QEA™ embodiment amplifies the cDNA sample prior to the RE/ligase reactions, removes unwanted fragments with a removal means, and then separates and detects the reaction products. Alternately, further amplification of the fragments of interest can be performed after the RE/ligase step.

In this embodiment, first, double stranded cDNA, perhaps prepared from a tissue sample according to Sec. 6.3.1, is PCR amplified using primers a conjugated capture moiety, preferably biotin. Any suitable primers known in the art, all biotin-labeled, can be used. For example, a set of arbitrary primers with no net sequence preference can be used. For a further example, where the cDNA is synthesized according to the protocol of Sec. 6.3.3, the method of step 6 of that protocol can be used, except that both the MA24 and MB24 have a conjugated biotin. The resulting cDNA with biotin linked to both ends is then cut with one or more REs and ligated to adapters corresponding to the REs used. The adapter primers can be optionally labeled but cannot have a conjugated biotin. The RE/ligase reaction is preferably performed according to the protocols of Sec. 5.2.2 in order that the RE digestion and adapter ligation proceed to completion with minimum formation of concatamers and other unwanted ligation products. The reaction products comprise fragments of interest that are doubly cut by REs and without any conjugated biotin, and unwanted fragments with a biotin conjugated to one end that are singly cut and derive from the

ends of cDNAs. Next, the unwanted singly cut fragments are removed by contacting the reaction products with streptavidin beads. Optionally, the purified fragments of interest can be blunt-ended and subject to further PCR amplification for a minimum number of cycles to observe the signals of interest. Finally, the products are then analyzed, also as in the prior sections, by separation according to length and by detection of the DNA and of the optionally labeled adapter primers, which indicate the RE cutting each fragment.

10 Other direct removal means may alternatively be used in this invention. Such removal means include but are not limited to digestion by single strand specific nucleases or passage though a single strand specific chromatographic column, for example, containing hydroxyapatite.

15 It will be apparent to those of skill in the art, that these alternative protocols using cDNAs with a conjugated capture moiety can combined with the other QEA™ embodiments in various manners. This invention encompasses all such insubstantially different variations.

20

5.3. PCR EMBODIMENT OF QEA™

An alternative implementation of QEA™ methods not using REs is based on PCR, or alternative amplification means, to select and amplify cDNA fragments between chosen 25 target subsequences recognized by amplification primers.

See, generally, Innis et al., 1989, PCR Protocols A Guide to Methods and Applications, Academic Press, New York, and Innis et al., 1995, PCR Strategies, Academic Press, New York.

Typically target subsequences between four and 30 eight base pairs long chosen by the methods previously described are preferred because of their greater probability of occurrence, and hence information content, as compared to longer subsequences. However, DNA oligomers this short may not hybridize reliably and reproducibly to their 35 complementary subsequences to be effectively used as PCR primers. Hybridization reliability depends strongly on several variables, including primer composition and length,

stringency condition such as annealing temperature and salt concentration, and cDNA mixture complexity. For the hash code to be effective for gene calling, it is highly preferred that subsequence recognition be as specific and reproducible 5 as possible so that well resolved bands representative only of the underlying sample sequence are produced. Thus, instead of directly using single short oligonucleotides complementary to the selected, target subsequences as primers, it is preferable to use carefully designed primers.

10 The RE embodiments of QEA™ have been verified to produce reproducible signal patterns over a 10³ range on input DNA concentrations. The PCR embodiment is less preferred because the input DNA concentration, as well as the initial hybridization temperature, must be closely to yield 15 reproducible results.

The preferred primers are constructed according to the model in Fig. 5. Primer 501 is constructed of three components, which, listed 5' to 3', are 504, 503, and 502. Component 503, described *infra*, is optional. Component 502 20 is a sequence which is complementary to the subsequence which primer 501 is designed to recognize. Component 502 is typically 4-8 bp long. Component 504 is a 10-20 bp sequence chosen so the final primer does not hybridize with any native sequence in the cDNA sample to be analyzed; that is, primer 25 501 does not anneal with any sequence known to be present in the sample to be analyzed. The sequence of component 504 is also chosen so that the final primer has a melting point above 50°C, and preferably above 68°C. The method for controlling melting temperature selecting average primer 30 composition and primer length is described above.

Use of primer 501 in the PCR embodiment involves a first annealing step, which allows the 3' end component 502 to anneal to its target subsequence in the presence of end component 504, which may not hybridize. Preferably, this 35 annealing step is at a temperature between 36 and 44°C that is empirically determined to maximize reproducibility of the resulting signal pattern. The DNA concentration is

approximately 10 ng/50 ml and is similarly determined to maximize reproducibility. Other PCR conditions are standard and are described in Sec. 6.6. Once annealed, the 3' end serves as the primer elongation point for the subsequent 5 first elongation step. The first elongation step is preferably at 72°C for 1 minute.

If stringency conditions are such that exact complementarity is not required for hybridization, false positive signals can be generated, that is signals resulting 10 from inexact recognition of the target subsequence. The generation of these false positive bands can be accounted for in the experimental analysis methods in order that DNA sample sequences can still be recognized, but, perhaps, with some increased recognition ambiguity that may need resolution. 15 These bands are accounted for by allowing inexact hybridization matches of the target subsequence, the degree of inexactness depending on the stringency of the hybridization conditions. In this case the signals generated contain only a fuzzy representation of the actual subsequence 20 in the sample, the degree of fuzziness being a function of subsequence length and the stringency condition, that is binding free energy, and the temperature of the hybridization. Given the free energy and temperature, the various possible actual subsequences can be approximately 25 determined by well known thermodynamic equilibrium calculations.

Subsequent PCR cycles then use high temperature, high stringency annealing steps. The high stringency annealing steps ensure exact hybridization of the entire 30 primer. No further false positive bands are generated. Preferably, these PCR cycles alternate between a 65°C annealing step and 95°C melting step, each for 1 minute.

Optional component 503 can be used to improve the specificity of the first low stringency annealing step and 35 thereby minimize false positive bands generated then. Component 503 can be -(N)_j-, where N is any nucleotide and j is typically between 2 and 4, preferably 2. Use of all

possible components 503 results in a degenerate set of primers, 16 primers if $j=2$, which have a 3' end subsequence effectively j bases longer than the target subsequence. These longer complementary end sequences have improved 5 hybridization specificity. Alternately, component 503 can be $-(U)_j-$, where N is a "universal" nucleotide and j is typically between 2 and 4, preferably 3 or 4. A universal nucleotide, such as inosine, is capable of forming base pairs with any other naturally occurring nucleotide. In this alternative, 10 single primer 501 has a 3' end subsequence effectively j bases longer than the target, and thus also has improved hybridization specificity.

A less preferred primer design comprises sets of degenerate oligonucleotides of sufficient length to achieve 15 specific and reproducible hybridization, where each member of a set includes a shared subsequence complementary to one selected, target sequence. For example, if a subsequence to be recognized is GATT, the set of primers used may be all sequences of the form NNAATCNN, where N is any nucleotide.

20 Also sets of degenerate primers permit the recognition of discontinuous subsequences. For example, GA--TT may be recognized by all sequences of the form NAANNTCNN. Alternately, a universal nucleotide can be used in place of the degenerate nucleotides represented by 'N'.

25 Each primer or primer set used in a single reaction is preferably distinctively labeled for detection. In the preferred embodiment using electrophoretic fragment separation, labeling is by fluorochromes that can be simultaneously distinguished with optical detection means.

30 An exemplary experimental protocol is summarized here, with details presented in Sec. 6.6. Total cellular mRNA or purified sub-pools of cellular mRNA are used for cDNA synthesis. First strand cDNA synthesis is performed according to Sec. 6.3 using, for example, an oligo(dT) primer 35 or alternatively phasing primers. Alternatively, cDNA samples can be prepared from any source or be directly obtained.

Next, using a first strand cDNA sample, the primers of the selected primer sets are used in a conventional PCR amplification protocol. A high molar excess of primers is preferably used to ensure only fragments between primer sites 5 that are adjacent on a target cDNA sequence or gene are amplified. With a high molar excess of primers binding to all available primer binding sites, no amplified fragment should include internally any primer recognition site. As many primers can be used in one reaction as can be labeled 10 for concurrent separation and detection and which generate an adequately resolved length distribution, as in the RE embodiments. For example, if fluorochrome labeling is used, each pair of fluorochromes preferably is distinguishable in one band and separate pairs preferably are distinguishable in 15 separate bands. After amplification, the fragments are separated, re-suspended for gel electrophoresis, electrophoretically separated, and optically detected. Thereby the length distribution of fragments having 20 particular pairs of target subsequences at their ends is ascertained.

Preferred protocols for the specific PCR embodiments are described in detail in Sec. 6.6.

5.4. QEA™ ANALYSIS AND DESIGN METHODS

25 This invention provides two groups of methods for the Quantitative Expression Analysis embodiment of this invention: first, methods for QEA™ experimental design; and second, methods for QEA™ experimental analysis. Although, logically, design precedes analysis, the methods of 30 experimental design depend on basic methods described herein as part of experimental analysis. Consequently, experimental analysis methods are described first.

In the following, descriptions are often cast in terms of the preferred QEA™ embodiment, in which REs are used 35 to recognize target subsequences. However, such description is not limiting, as all the methods to be described are equally adaptable to all QEA™ embodiments, including those in

which target subsequences are recognized by nucleic acid, or nucleic acid mimic, and probes which recognize target subsequences by hybridization.

Further, the following descriptions are directed to 5 the currently preferred embodiments of these methods.

However, it will be readily apparent to those skilled in the computer and simulation arts that many other embodiments of these methods are substantially equivalent to those described and can be used to achieve substantially the same results.

10 This invention comprises such alternative implementations as well as its currently preferred implementation.

5.4.1. QEA™ EXPERIMENTAL ANALYSIS METHODS

The analysis methods comprise, first, selecting a 15 database of DNA sequences representative of the DNA sample to be analyzed, second, using this database and a description of the experiment to derive the pattern of simulated signals, contained in a database of simulated signals, which will be produced by DNA fragments generated in the experiment, and 20 third, for any particular detected signal, using the pattern or database of simulated signals to predict the sequences in the original sample likely to cause this signal. Further analysis methods present an easy to use user interface and permit determination of the sequences actually causing a 25 signal in cases where the signal may arise from multiple sequences, and perform statistical correlations to quickly determine signals of interest in multiple samples.

The first analysis method is selecting a database of DNA sequences representative of the sample to be analyzed.

30 In the preferred use of this invention, the DNA sequences to be analyzed will be derived from a tissue sample, typically a human sample examined for diagnostic or research purposes.

In this use, database selection begins with one or more publicly available databases which comprehensively record all 35 observed DNA sequences. Such databases are GenBank from the National Center for Biotechnology Information (Bethesda, MD), the EMBL Data Library at the European Bioinformatics

Institute (Hinxton Hall, UK) and databases from the National Center for Genome Research (Santa Fe, NM). However, as any sample of a plurality of DNA sequences of any provenance can be analyzed by the methods of this invention, any database 5 containing entries for the sequences likely to be present in such a sample to be analyzed is usable in the further steps of the computer methods.

Fig. 6A illustrates the preferred database selection method starting from a comprehensive tissue derived 10 database. Database 1001 is the comprehensive input database, having the exemplary flat-file or relational structure 1010 shown in Fig. 6B, with one row, or record, 1014 for each entered DNA sequence. Column, or field, 1011 is the accession number field, which uniquely identifies each 15 sequence in database 1001. Most such databases contain redundant entries, that is multiple sequence records are present that are derived from one biological sequence. Column 1013 is the actual nucleotide sequence of the entry. The plurality of columns, or fields, represented by 1012 20 contain other data identifying this entry including, for example whether this is a cDNA or gDNA sequence, if cDNA, whether this is a full length coding sequence or a fragment, the species origin of the sequence or its product, the name of the gene containing the sequence, if known, etc. Although 25 shown as one file, DNA sequence databases often exists in divisions and selection from all relevant divisions is contemplated by this invention. For example, GenBank has 15 different divisions, of which the EST division and the separate database, dbEST, that contain expressed sequence 30 tags ("EST") are of particular interest, since they contain expressed sequences.

From the comprehensive database, all records are selected which meet criteria for representing particular experiments on particular tissue types. This is accomplished 35 by conventional techniques of sequentially scanning all records in the comprehensive database, selecting those that

match the criteria, and storing the selected records in a selected database.

The following are exemplary selection methods. To analyze a genomic DNA sample, database 1001 is scanned 5 against criteria 1002 for human gDNA to create selected database 1003. To analyze expressed genes (cDNA sequences), several selection alternatives are available. First, a genomic sequence can be scanned in order to predict which subsequences (exons) will be expressed. Thus selected 10 database 1005 is created by making selections according to expression predictions 1004. Second, observed expressed sequences, such as cDNA sequences, coding domain sequences ("CDS"), and ESTs, can be selected 1006 to create selected database 1007 of expressed sequences. Additionally, 15 predicted and observed expressed sequences can be combined into another, perhaps more comprehensive, selected database of expressed sequences. Third, expressed sequences determined by either of the prior methods may be further selected by any available indication of interest 1008 in the 20 database records to create more targeted selected database 1009. Without limitation, selected databases can be composed of sequences that can be selected according to any available relevant field, indication, or combination present in sequence databases.

25 The second analysis method uses the previously selected database of sequences likely to be present in a sample and a description of an intended experiment to derive a pattern of the signals which will be produced by DNA fragments generated in the experiment. This pattern can be 30 stored in a computer implementation in any convenient manner. In the following, without limitation, it is described as being stored as a table of information. This table may be stored as individual records or by using a database system, such as any conventionally available relational database. 35 Alternatively, the pattern may simply be stored as the image of the in-memory structures which represent the pattern.

A QEA™ experiment comprises several independent recognition reactions applied to the DNA sample sequences, where in each of the reactions labeled DNA fragments are produced from sample sequences, the fragments lying between 5 certain target subsequences in a sample sequence. The target subsequences can be recognized and the fragments generated by the preferred RE embodiments of QEA™ methods or by the PCR embodiment of QEA™. The following description is focused on the RE embodiments.

10 Fig. 7 illustrates an exemplary description 1100 of a preferred QEA™ embodiment. Field 1101 contains a description of the tissue sample which is the source of the DNA sample. For example, one experiment could analyze a normal prostate sample; a second otherwise identical 15 experiment could analyze a prostate sample with premalignant changes; and a third experiment could analyze a cancerous prostate sample. Differences in gene expression between these samples then relate to the progress of the cancer disease state. Such samples could be drawn from any other 20 human cancer or malignancy.

Major rows 1102, 1105, and 1109 describe the separate individual recognition reactions to which the DNA from tissue sample 1101 is subjected. Any number of reactions may be assembled into an experiment, from as few as 25 one to as many as there are pairs of available recognition means to recognize subsequences. Fig. 7 illustrates 15 reactions. For example, reaction 1 specified by major row 1102 generates fragments between target subsequences which are the recognition sites of restriction endonucleases 1 and 30 2 described in minor rows 1103 and 1104. Further, the RE1 cut end is recognized by a labeling moiety labeled with LABEL1, and the RE2 end is recognized by LABEL2. Similarly, reaction 15, 1109, utilizes restriction endonucleases 36 and 35 labeled with labels 3 and 4, minor rows 1110 and 1111, respectively.

Major row 1105 describes a variant QEA™ reaction using three REs and a separate probe. As described, many REs

can be used in a single recognition reaction as long as a useful fragment distribution results. Too many REs results in a compressed length distribution. Further, probes for target subsequences that are not intended to be labeled 5 fragment ends, but rather occur within a fragment, can be used. For example, a labeled probe added after QEA™ PCR amplification step (if present in a given embodiment), a post PCR probe, can recognize subsequences internal to a fragment and thereby provide an additional signal which can be used to 10 discriminate between two sample sequences which produce fragments of the same length and end sequence which otherwise have differing internal sequences. For another example, a probe added before QEA™ PCR step and which cannot be extended by DNA polymerase will prevent PCR amplification of those 15 fragment containing the probe's target subsequences. If PCR amplification is necessary to generate detectable signals (in a given embodiment), such a probe will prevent the detection of such a fragment. The absence of a fragment may make a previously ambiguous detected band now unambiguous. Such PCR 20 disruption probes can be PNA oligomers or degenerate sets of DNA oligomers, modified to prevent polymerase extension (e.g., by incorporation of a dideoxynucleotide at the 3' end).

Where alternative phasing PCR primers are used, 25 their extra recognition subsequences and labeling are described in rows dependent to the RE/ligase reaction whose products they are used to amplify.

Next Fig. 8A illustrates, in general, that from the database selected to best represent the likely DNA sequences 30 in the sample analyzed, 1201, and the description of QEA™ experiment, 1202, the simulation methods, 1203, determine a pattern of simulated signals stored in a simulated database, 1204, that represents the results of QEA™ experiments. The experimental simulation generates the same fragment lengths 35 and end subsequences from the input database that will be generated in an actual experiment performed on the same sample of DNA sequences.

Alternately, the simulated pattern or database may not be needed, in which case the DNA database is searched sequence by sequence, mock digestions are performed and compared against the input signals. A simulated database is 5 preferable if several signals need to be searched or if the same QEA™ experiment is run several times. Conversely, the simulated database can be dispensed with when few signals from a few experiments need to be searched. A quantitative statement of when the simulated database is more efficient 10 depends upon an analysis of the costs of the various operations and the size of DNA database, and can be performed as is well known in the computer arts. Without limitation, in the following the simulated database is described

Fig. 8B illustrates an exemplary structure for the 15 simulated database. Here, the simulated results of all the individual recognition reactions defined for the experiment are gathered into rectangular table 1210. The invention is equally adaptable to other database structures containing equivalent information; such an equivalent structure would be 20 one, for example, where each reaction was placed in a separate table. The rows of table 1210 are indexed by the lengths of possible fragments. For example, row 1211 contains fragments of length 52. The columns of table 1210 are indexed by the possible end subsequences and probe hits, 25 if any, in a particular experimental reaction. For example, columns 1212, 1213, and 1214 contain all fragments generated in reaction 1, R1, which have both end subsequences recognized by RE1, one end subsequence recognized by RE1 and the other by RE2, and both end subsequences recognized by 30 RE2, respectively. Other columns relate to other reactions of the experiment. Finally, the entries in table 1210 contain lists of the accession numbers of sequences in the database that give rise to a fragment with particular length and end subsequences. For example, entry 1215 indicates that 35 only accession number A01 generates a fragment of length 52 with both end subsequences recognized by RE1 in R1. Similarly, entry 1216 indicates that accession numbers A01

and S003 generate a fragment of length 151 with both end subsequences recognized by RE3 in reaction 2.

In alternative embodiments, the contents of the table can be supplemented with various information. In one aspect, this information can aid in the interpretation of results produced by the separation and detection means used. For example, if separation is by electrophoresis, then the detected electrophoretic DNA length can be corrected to obtain the true physical DNA length. Such corrections are well known in the electrophoretic arts and depend on such factors as average base composition and fluorochrome labels. One commercially available package for making these corrections is Gene Scan Software from Applied Biosystems, Inc. (Foster City, CA). In this case, each table entry for a fragment can contain additionally average base composition, perhaps expressed as percent G+C content, and the experimental definition can include primer average base composition and fluorochrome label used. For a further example, if separation is by mass spectroscopy or similar method, the additional information can be the molecular weight of each fragment and perhaps a typically fragmentation pattern. Use of other separation and detection means can suggest the use of other appropriate supplemental data.

Where alternative phasing primers, the SEQ-QEA™ embodiment, or other means generating effective target subsequences are used, supplemental columns are used with RE pair in order to further identify such effective target subsequence.

Before describing how this simulated database is generated, it is useful first to describe how this database is used to predict experimental results. Returning to Fig. 7, labels are used to detect binding reaction events by subsequence recognition means to the target DNA, to allow detection after separation of the fragments by length. In an embodiment using fluorescent detection means, these labels are fluorochromes covalently attached to the primer strands of the adapters, as previously described, or to hybridization

probes, if any. Typically, all the fluorochrome labels used in one reaction are simultaneously distinguishable so that fragments with all possible combinations of target subsequences can be fluorescently distinguished. For example, fragments at entry 1217 in table 1210 (Fig. 8B) occur at length 175 and present simultaneous fluorescent signals LABEL1 and LABEL2 upon stimulation, since these are the labels used with adapters which recognize ends cuts by RE1 and RE2 respectively. For a further example, in reaction 10 2, major row 1105 of experimental definition 1100 (Fig. 7), a fragment with ends cut by RE2 and RE3 and hybridizing with probe P will present simultaneous signals LABEL2, LABEL3, and LABEL4. Where effective target subsequences are constructed, e.g. by SEQ-QEA™ or alternative phasing primers, this lookup 15 is appropriately modified.

Other labelings are within the scope of this invention. For example, a certain group of target subsequences can be identically labeled or not labeled at all, in which case the corresponding group of fragments are 20 not distinguishable. In this case, if RE1 and RE3 end subsequences were identically labeled in table 1210 (Fig. 8B), a fragment of length 151 may be generated by sequence T162, A01, or S003, or any combination of these sequences. In the extreme, if silver (Ag) staining of an electrophoresis 25 gel is used in an embodiment to detect separated fragments, then all bands will be identically labeled and only band lengths can be distinguished within one electrophoresis lane.

Thus the simulated database together with the experimental definition can be used to predict experimental 30 results. If a signal is detected in a recognition reaction, say Rn, whose end labelings are LABEL1 and LABEL2 and whose representation of length is corrected to physical length in base pairs of L, the length L row of the simulated database is retrieved and it is scanned for Rn entries with the 35 detected subsequence labeling, by using the column headings indicating observed subsequences and the experimental definition indicating how each subsequence is labeled. If no

match is found, this fragment represents a new gene or sequence not present in the selected database. If a match is found, then this fragment, in addition to possibly being a new gene or sequence, can also have been generated by those 5 candidate sequences present in the table entry(ies) found.

The simulated database lookup is described herein as using the physical length of a detected fragment. In cases where the separation and detection means returns an approximation to the true physical fragment length, lookup is 10 augmented to account for such as approximation. For example, electrophoresis, when used as the separation means, returns the electrophoretic length, which depending on average base composition and labeling moiety is typically within 10% of the physical length. In this case database lookup can search 15 all relevant entries whose physical length is within 10% of the reported electrophoretic length, perform corrections to obtain electrophoretic length, and then check for a match with the detected signal. Alternative lookup implementations are apparent, one being to precompute the electrophoretic 20 length for all predicted fragments, construct an alternate table index over the electrophoretic length, and then directly lookup the electrophoretic length. Other separation and detection means can require corresponding augmentations to lookup to correct for their particular experimental biases 25 and inaccuracies. It is understood that where database lookup is referred to subsequently, either simple physical lookup or augmented lookup is meant as appropriate.

If matched candidate database sequences are found, then the selected database can be consulted to determine 30 other information concerning these sequences, for example, gene name, tissue origin, chromosomal location, etc. If an unpredicted fragment is found, this fragment can be optionally retrieved from the length separation means, cloned or sequenced, and used to search for homologues in a DNA 35 sequence database or to isolate or characterize the previously unknown gene or sequence. In this manner this

invention can be used to rapidly discover and identify new genes.

The computer methods of this invention are also adaptable to other formats of an experimental definition.

5 For example, the labeling of the target subsequence recognition moieties can be stored in a table separate from the table defining the experimental reactions.

Now turning to the methods by which the simulated database is generated, Fig. 9 illustrates a basic method, 10 termed herein mock fragmentation, which takes one sequence and the definition of one reaction of an experiment and produces the predicted results of the reaction on that sequence. Generation of the entire simulated database requires repetitive execution of this basic method.

15 Turning first to a description of mock fragmentation, the method commences at 1301 and at 1302 it inputs the sequence to be fragmented and the definition of the fragmentation reaction, in the following terms: the target end subsequences RE1 ... RE_n where n is typically 2 20 or 3, and the subsequences to be recognized by post PCR probes, P1 ... P_n, where n is typically 0 or 1. Note that PCR disruption probes act as unlabeled end subsequences and are so treated for input to this method. The operation of the method is illustrated by example in Fig. 10A-F for the 25 case RE1, RE2 and P1.

At step 1303, for each target end subsequence, the method makes a "vector of ends", which has elements which are pairs of nucleotide positions along the sequence, each pair being labeled by the corresponding end subsequence. For 30 embodiments where end subsequences are recognized by hybridizing oligonucleotides, the first member of each pair is the beginning of a target end subsequence and the second member is the end of a target end subsequence. For embodiments where target end subsequences are recognized by 35 restriction endonucleases, the first member of each pair is the beginning of the overhang region that corresponds to the RE recognition subsequence and the second member is the end

of that overhang region. It is preferred to use REs that generate 4 bp overhangs. The actual target end subsequences are the RE recognition sequences, which are preferably 4-8 bp long.

5 This vector is generated by a string operation which compares the target end subsequence in a 5' to 3' direction against the input sequence and seeks string matches, that is the nucleotides match exactly. Where effective target subsequences are formed by using, e.g. SEQ-
10 QEA™ or alternative phasing primers, it is the effective subsequences that are compared. This can be done by simply comparing the end subsequence against the input sequence starting at one end and proceeding along the sequence one base at time. However, it is preferable to use a more
15 efficient string matching algorithm, such as the Knuth-Morris-Pratt or the Boyer-Moore algorithms. These are described with sample code in Sedgewick, 1990, Algorithms in C, chap. 19, Addison-Wesley, Reading, MA.

In QEA™ embodiments where target subsequence are
20 recognized with accuracy, such as the RE embodiments, the comparison of target subsequence against input sequence should be exact, that is the bases should match in a one-to-one manner. In embodiments where target subsequences are less accurately recognized, the string match should be done
25 in a less exact, or fuzzy, manner. For example, in the PCR embodiments, a target subsequence of length T can inaccurately recognize an input sequence, also of length T, by matching only T-n bases exactly, where n is typically 1 or 2 and is adjustable depending on experimental conditions. In
30 this case the string operation, which generates the vector of ends, should accept partial T-n matches as well as exact matches. In this, the string operations generate the false positive matches expected from the experiments and permit these fragments to be identified. Ambiguity in the simulated
35 database, however, increases, since more fragments leads to a greater chance of fragments of identical length and end labels.

Fig. 10A illustrates end vectors 1401 and 1402, comprising three and two ends, respectively, generated by RE1 and RE2, which are for this example assumed to be REs with a 4 bp overhang. The first overhang in vector 1401 occurs 5 between nucleotide 10 and 14 in the input sequence.

Step 1304 of Fig. 9 merges all the end vectors for all the end subsequences and sorts the elements on the position of the end. Vector 1404 of Fig. 10B illustrates the result of this step for example end vectors 1401 and 1402.

10 Step 1305 of Fig. 9 then creates the fragments generated by the reaction by selecting the parts of the full input sequence that are delimited by adjacent ends in the merged and sorted end vector. Since the experimental conditions in conducting QEA™ should be selected such that 15 target end subsequence recognition is allowed to go to completion, all possible ends are recognized. For the restriction endonuclease embodiments, the cutting and ligase reactions should be conducted such that all possible RE cuts are made and to each cut end a labeled primer is ligated.

20 These conditions insure that no fragments contain internal unrecognized target end subsequences and that only adjacent ends in the merged and sorted vector define generated fragments.

Where additional information is needed for 25 simulated database entries to adapt to inaccuracies in particular separation and detection means, such information can be collected at this step. For example, in the case of electrophoretic separation, fragment sequence can be determined and percent G+C content computed and entered in 30 the database along with the fragment accession number.

For the PCR embodiments, the fragment length is the difference between the end position of the second end subsequence and the start position of the first end subsequence. For RE embodiments, the fragment length is the 35 difference between the start position of the second end subsequence and the start position of the first end

subsequence plus twice the primer length (48 in the preferred primer embodiment).

Fig. 10C illustrates the exemplary fragments generated, each fragment being represented by a 4 member tuple comprising: the two end subsequences, the length, and an indicator whether the probe binds to this fragment. In Fig. 10C the position of this indicator is indicated by a '*'. Fragment 1408 is defined by ends 1405 and 1406, and fragment 1409 by ends 1406 and 1407. There is no fragment 10 defined by ends 1405 and 1407 because the intermediate end subsequence is recognized and either fully cut in an RE embodiment or used as a fragment end priming position in a PCR embodiment. For simplicity, the fragment lengths are illustrated for the RE embodiment without the primer length 15 addition.

Step 1306 of Fig. 9 checks if a hybridization probe is involved in the experiment. If not, the method skips to step 1309. If so, step 1307 determines the sequence of the fragment defined in step 1305. Fig. 10D illustrates that the 20 fragment sequences for this example are the nucleotide sequences within the input sequence that are between the indicated nucleotide positions. For example, the first fragment sequence is the part of the input sequence between positions 10 and 62. Step 1308 then checks each probe 25 subsequence against each fragment sequence to determine whether there is any match (i.e., whether the probe has a sequence complementary enough to the fragment sequence sufficient for it to hybridize thereon). If a match is found, an indication is made in the fragment 4 member tuple. 30 This match is done by string searching in a similar manner to that described for generation of the end vectors.

Next at step 1309 of Fig. 9, all the fragment are sorted on length and assembled into a vector of sorted fragments, which is output from the mock fragmentation method 35 at step 1310. This vector contains the complete list of all fragments, with probe information, defined by their end

subsequences and lengths that the input reaction will generate from the input sequence.

Fig. 10E illustrates the fragment vector of the example sorted according to length. For illustrative purposes, probe P1 was found to hybridize only to the third fragment 1412, where a 'Y' is marked. 'N' is marked in all the other fragments, indicating no probe binding.

The simulated database is generated by iteratively applying the basic mock fragmentation method for each sequence in the selected database and each reaction in the experimental definition. Fig. 11 illustrates a simulated database generation method. The method starts at 1501 and at 1502 inputs the selected representative database and the experimental definition with, in particular, the list of reactions and their related subsequences. Step 1503 initializes the digest database table so that lists of accession numbers may be inserted for all possible combinations of fragment length and target end subsequences. Step 1504, a DO loop, causes the iterative execution of steps 1505, 1506, and 1507 for all sequences in the input selected database.

Step 1505 takes the next sequence in the database, as selected by the enclosing DO loop, and the next reaction of the experiment and performs the mock fragmentation method of Fig. 9, on these inputs. Step 1506 adds the sorted fragment vector to the simulated database by taking each fragment from the vector and adding the sequence accession number to the list in the database entry indexed by the fragment length and end subsequences and probe (if any). Fig. 10F represents the simulated database entry list additions that would result for the example mock fragmentation reaction of Figs. 10A-E. For example, accession number A01 is added to the accession number list in the entry 1412 at length 151 and with both end subsequences RE2.

Finally, step 1507 tests whether there is another reaction in the input experiment that should be simulated

against this sequence. If so, step 1505 is repeated with this reaction. If not, the DO loop is repeated to select another database sequence. If all the database sequences have been selected, the step 1508 outputs the simulated 5 database and the method ends at 1509.

5.4.2. QEA™ EXPERIMENTAL DESIGN METHODS

The goal of the experimental design methods is to optimize each experiment in order to obtain the maximum 10 amount of quantitative information. An experiment is defined by its component recognition reactions, which are in turn defined by the target end subsequences recognized, probes used, if any, and labels assigned. If alternative phasing primers, SEQ-QEA™, or other similar means are used, effective 15 target subsequences are used. Any of several criteria can be used to ascertain the amount of information obtained, and any of several algorithms can be used to perform the reaction optimization.

A preferred criteria for ascertaining the amount of 20 information uses the concept of "good sequence." A good sequence for an experiment is a sequence for which there is at least one reaction in the experiment that produces a unique signal from that sequence, that is, a fragment is produced from that good sequence, by at least one recognition 25 reaction, that has a unique combination of length and labeling. For example, returning to Fig. 8B, the sequence with accession number A01 is a good sequence because reaction 1 produces signal 1215, with length 52 and with both target end subsequences recognized by RE1, uniquely from sequence 30 A01. However, sequence S003 is not a good sequence because there are no unique signals produced only from S003: reaction R2 produces signal 1216 from both A01 and S003 and signal 1219 from both Q012 and S003. Using the amount of good sequences as an information measure, the greater the number 35 of good sequences in an experiment the better is the experimental design. Ideally, all possible sequences in a sample would be good sequences.

Further, a quantitative measure of the expression of a good sequence can simply be determined from the detected signal intensity of the fragment uniquely produced from the good sequence. Relative quantitative measures of the expression of different good sequences can be obtained by comparing the relative intensities of the signal uniquely produced from the good sequences. An absolute quantitative measure of the expression of a good sequence can be obtained by including a concentration standard in the original sample. Such a standard for a particular experiment can consist of several different good sequences known not to occur in the original sample and which are introduced at known concentrations. For example, exogenous good sequence 1 is added at a 1:10³ concentration in molar terms; exogenous good sequence 2 at a 1:10⁴ in molar terms; etc. Then comparison of the relative intensity of the unique signal of a good sequence in the sample with the intensities of the unique signal of the standards allows determination of the molar concentrations of the sample sequence. For example, if the good sequence has a unique signal intensity half way between the unique signal intensities of good sequences 1 and 2, then it is present at a concentration half way between the concentrations of good sequences 1 and 2.

Another preferred measure for ascertaining the amount of information produced by an experiment is derived by limiting attention to a particular set of sequences of interest, for example a set of known oncogenes or a set of receptors known or expected to be present in a particular tissue sample. An experiment is designed according to this measure to maximize the number of sequences of interest that are good sequences. Whether other sequences possibly present in the sample are good sequences is not considered. These other sequences are of interest only to the extent that the sequences of interest produce uniquely labeled fragments without any contribution from these other sequences.

This invention is adaptable to other measures for ascertaining information from an experiment. For example,

another measure is to minimize on average the number of sequences contributing to each detected signal. A further measure is, for example, to minimize for each possible sequence the number of other sequences that occur in common in the same signals. In that case each sequence is linked by common occurrences in fragment labelings to a minimum number of other sequences. This can simplify making unambiguous signal peaks of interest (see *infra*).

Having chosen an information measure, for example 10 the number of good sequences, for an experiment, the optimization methods choose target subsequences, and possibly probes, which optimize the chosen measure. One possible optimization method is exhaustive search, in which all subsequences in lengths less than approximately 10 are tested 15 in all combinations for that combination which is optimum. This method requires considerable computing power, and the upper bound is determined by the computational facilities available and the average probability of occurrence of subsequences of a given length. With adequate resources, it 20 is preferable to search all sequences down to a probability of occurrence of about 0.005 to 0.01. Upper bounds may range from 8 to 11 or 12.

A preferred optimization method is known as simulated annealing. See Press et al., 1986, Numerical 25 Recipes - The Art of Scientific Computing, Sec. 10.9, Cambridge University Press, Cambridge, U.K. Simulated annealing attempts to find the minimum of an "energy" function of the "state" of a system by generating small changes in the state and accepting such changes according to 30 a probabilistic factor to create a "better" new state. While the method progresses, a simulated "temperature", on which the probabilistic factor depends and which limits acceptance of new states of higher energy, is slowly lowered.

In the application to the methods of this 35 invention, a "state", denoted by S, is the experimental definition, that is the target end subsequences and hybridization probes, if any, in each recognition reaction of

the experiment. The "energy", denoted E , is taken to be 1.0 divided by the information measure, so that when the energy is minimized, the information is maximized. Alternatively, the energy can be any monotonically decreasing function of 5 the information measure. The computation of the energy is denoted by applying the function $E()$ to a state.

The preferred method of generating a new experiment, or state, from an existing experiment, or state, is to make the following changes, also called moves to the 10 experimental definition: (1) randomly change a target end subsequence in a randomly chosen recognition reaction; (2) add a randomly chosen target end subsequence to a randomly chosen reaction; (3) remove a randomly chosen target end subsequence from a randomly chosen reaction with three or 15 more target subsequences; (4) add a new reaction with two randomly chosen target end subsequences; and (5) remove a randomly chosen reaction. If an RE embodiment of QEA™ is being designed, all target end subsequences are limited to available RE recognition sequences. If alternative phasing 20 primers, SEQ-QEA™, or other means are used to generate effective target subsequences, all subsequences must be chosen from among such effective target subsequences that can be generated from available REs. To generate a new experimental definition, one of these moves is randomly 25 selected and carried out on the existing experimental definition. Alternatively, the various moves can be unequally weighted. In particular, if the number of reactions is to be fixed, moves (4) and (5) are skipped. The invention is further adaptable to other moves for generating 30 new experiments. Preferable generation methods will generate all possible experiments.

Several additional subsidiary choices are needed in order to apply simulated annealing. The "Boltzman constant" is taken to be 1.0, so that the energy equals the 35 temperature. The minimum of the energy and temperature, denoted E_0 and T_0 , respectively, are defined by the maximum of the information measure. For example, if the number of good

sequences of interest is G and is used as the information measure, then E_0 , which equals T_0 , equals $1/G$. An initial temperature, denoted T_1 , is preferably chosen to be 1. An initial experimental definition, or state, is chosen, either 5 randomly or guided by prior knowledge of previous experimental optimizations. Finally, two execution parameters are chosen. These parameters define the "annealing schedule", that is the manner in which the temperature is decreased during the execution of the 10 simulated annealing method. They are the number of iterations in an epoch, denoted by N , which is preferably taken to be 100 and the temperature decay factor, denoted by f , which is preferably taken to be 0.95. Both N and f may be systematically varied case-by-case to achieve a better 15 optimization of the experiment definition with a lower energy and a higher information measure.

With choices for the information measure or energy function, the moves for generating new experiments, an initial state or experiment, and the execution parameters 20 made as above, the general application of simulated annealing to optimize an experimental definition is illustrated in Fig. 13A. The information measure used in this description is the number of good sequences of interest. Any information measure, such as those previously described, may be used 25 alternately.

The method begins at step 1701. At step 1702 the temperature is set to the initial temperature; the state to the initial state or experimental definition; and the energy is set to the energy of the initial state. At step 1703 the 30 temperature and energy are checked to determine whether either is less than or equal to the minima for the information measure chosen, as the result of either a fortuitous initial choice or subsequent computation steps. If the energy is less than or equal to the minimum energy, no 35 further optimization is possible, and the final experimental definition and its energy is output. If the temperature is less than or equal to the minimum temperature, the

optimization is stopped. Then the inverse of the energy is the number of good sequences of interest for this experimental definition.

Step 1706 is a DO loop which executes an epoch, or 5 N iterations, of the simulated annealing algorithm. Each iteration consists of steps 1707 through 1711. Step 1707 generates a new experimental definition, or state, S_{new} , according to the described generation moves. Step 1708 ascertains or determines the information content, or energy, 10 of S_{new} . Step 1709 tests the energy of the new state, and, if it is lower than the energy of the current state, at step 1711, the new state and new energy are accepted and replace the current state and current energy. If the energy of the new state is higher than the energy of the current state, 15 step 1710 computes the following function.

$$EXP[-(E-E_{new})/T]$$

This function defines the probabilistic factor controlling acceptance. If this function is less than a random chosen 20 number uniformly distributed between 0 and 1, then the new state is accepted at step 1711. If not, then the newly generated state is discarded. These steps are equivalent to accepting a new state if the energy is not increased by an amount greater than that determined by function (4) in 25 conjunction with the selection of a random number. Or in other words, a new state is accepted if the new information measure is not decreased by an amount greater than indirectly determined by function (4).

Finally, after an epoch of the algorithm, at step 30 1712 the temperature is reduced by the multiplicative factor f and the method loops back to the test at step 1703.

Using this algorithm, starting from an initial experimental definition which has certain information content, the algorithm produces a final experimental 35 definition with a higher information content, or lower energy, by repetitively and randomly altering the

experimental definition in order to search for a definition with a higher information content.

The computation of the energy of an experimental definition, or state, in step 1708 is illustrated more detail 5 in Fig. 13B. This method starts at step 1720. Step 1721 inputs the current experimental definition. Step 1722 determines a complete digest database from this definition and a particular selected database by the method of Fig. 11. Step 1723 scans the entire digest database and counts the 10 number of good sequences of interest. If the total number of good sequences is the measure used, the total number of good sequences can be counted. Alternatively, other information measures may be applied to the digest database. Step 1724 computes the energy as the inverse of the information 15 measure. Alternatively, another decreasing function of the information content may be used as the energy. Step 1725 outputs the energy, and the method ends at step 1726.

5.4.3. QEA™ AMBIGUITY RESOLUTION

20 In one utilization of this invention two related tissue samples can be subject to the same experiment, perhaps consisting of only one recognition reaction, and the outcomes compared. The two tissue samples may be otherwise identical except for one being normal and the other diseased, perhaps 25 by infection or a proliferative process, such as hyperplasia or cancer. One or more signals may be detected in one sample and not in the other sample. Such signals might represent genetic aspects of the pathological process in one tissue. These signals are of particular interest.

30 The candidate sequences that can produce a signal of interest are determined, as previously described, by look-up in the digest database. The signal may be produced by only one sequence, in which case it is unambiguously identified. However, even if the experiment has been 35 optimized, the signal may be ambiguous in that it may be produced by several candidate sequences from the selected

database. A signal of interest may be made unambiguous in several manners which are described herein.

In a first manner of making unambiguous assume the signal of interest is produced by several candidate sequences 5 all of which are good sequences for the particular experiment. Then which sequences are present in the signal of interest can be ascertained by determining the quantitative presence of the good sequences from their unique signals. For example, referring to Fig. 8B, if the signal 10 1217 of length 175 with the labeling 1213 is of interest, the sequences actually present in the signal can be determined from the quantitative determination of the presence of signals 1215 and 1218. Here, both the possible sequences contributing to this signal are good sequences for this 15 experiment.

The first manner of making unambiguous can be extended to the case where one of the sequences possibly contributing to a signal is not a good sequence. The quantitative presence of all the possible good sequences can 20 be determined from the quantitative strength of their unique signals. The presence of the remaining sequence which is not a good sequence can be determined by subtracting from the quantitative presence of the signal of interest the quantitative presences of all the good sequences.

25 Further extensions of the first manner can be made to cases where more than one of the possible sequences is not a good sequences if the sequences which are not good appear as contributors to further signals involving good sequences in a manner which allows their quantitative presences to be 30 determined. For example, suppose signal 1219 is of interest, where both possible sequences are not good sequences. The quantitative presence of sequence Q012 can be determined from signals 1220 and 1218 in the manner previously outlined. The quantitative presence of sequence S003 can be determined from 35 signals 1216 and 1215. Thereby, the sequences contributing to signal 1219 can be determined. More complex combinations can be similarly made unambiguous.

An alternative extension of the first manner of making unambiguous is by designing a further experiment in which the possible sequences contributing to a signal of interest are good sequences even if they were not originally so. Since there are approximately 50 suitable REs that can be used in the RE embodiment of QEA™ (Section 6.2), there are approximately 600 RE reaction pairs that can be performed, assuming that half of the theoretical maximum of 1,250 (50 x 50 / 2 = 1,250) are not useable. Since most RE pairs produce on the average of 200 fragments and standard electrophoretic techniques can resolve at least approximately 500 fragment lengths per lane, the RE QEA™ embodiment has the potential of generating over 100,000 signals (500 x 200 = 100,000). The number of possible signals is further increased by the use of reactions with three or more REs and by the use of labeled probes. Further, since the average complex human tissue, for example brain, is estimated to express no more than approximately 25,000 genes, there is a 4 fold excess of possible signals over the number of possible sequences in a sample. Thus it is highly likely that for any signal of interest, a further experiment can be designed and optimized for which all possible candidates of the signal of interest are good sequences. This design can be made by using the prior optimization methods with an information measure the sequences of interest in the signal of interest and starting with an extensive initial experimental definition including many additional reactions. In that manner, any signal of interest can be made unambiguous.

A second manner of making unambiguous is by automatically ranking the likelihood that the sequences possibly present in a signal of interest are actually present using information from the remainder of the experimental reactions. Fig. 14 illustrates a preferred ranking method. The method begins at step 1801 and at step 1802 inputs the list of possible accession numbers in a signal of interest, the experimental definition, and the actual experimental results. DO-loop 1803 iterates once for each possible

accession number. Step 1804 performs a simulated experiment by the method illustrated in Fig. 11 in which, however, only the current accession number is acted on. The output is a single sequence digest table, such as illustrated in Fig. 5 10F.

Step 1805 determines a numerical score of ranking the similarity of this digest table to the experimental results. One possible scoring metric comprises scanning the digest table for all fragment signals and adding 1 to the 10 score if such a signal appears also in the experimental results and subtracting 1 from the score if such signal does not appear in the experimental results. Alternate scoring metrics are possible. For example, the subtraction of 1 may be omitted.

15 Step 1806 sorts the numerical scores of the likelihood that each possible accession number is actually present in the sample. Step 1807 outputs the sorted list and the method ends at step 1808.

By this method likelihood estimates of the presence 20 of the various possible sequences in a signal of interest can be determined.

5.5. COLONY CALLING

The colony calling embodiment recognizes and 25 classifies single, individual genes or DNA sequences by determining the presence or absence of target subsequences. No length information is determined. This embodiment is directed to gene determination and classification of arrayed samples or colonies, where each sample or colony contains or 30 expresses only one sequence or gene of interest and is perhaps prepared from a tissue cDNA library. The presence or absence of target subsequences in a colony is determined by use of labeled hybridization recognition means, each of which uniquely binds to one target subsequence. It is preferable 35 that this binding be highly specific and reproducible. Each sample or colony, or an array of samples or colonies, is assayed for the contained sequence by determining which of

the set of probes recognizes and thus hybridizes to target subsequences in the sample(s) or colony(ies). Each sample is then characterized by a hash code, each bit of which indicates which probes recognized subsequences, or hits, in a particular sample. The sequence or gene in a sample is determined from the hash code by computer implemented methods.

The choice of the target subsequences is important. For economical and rapid assay, the size of the set of 10 recognition means should be as small as possible, preferably less than 50 elements and more preferably from 15 to 25 elements. Further, it is most preferable that all possible sequences or genes are recognized and uniquely determined. It is preferable that 90 to 95% of all possible sequences be 15 recognized, with each sequence being indistinguishable from, or ambiguous with, at most one or two other sequences. Therefore, each target subsequence preferably occurs frequently enough to minimize the number of different recognition means needed. For example, it is not practical 20 for this invention, directed to rapid gene classification, if each probe recognized only a few genes and therefore thousands of probes were needed. However, each target subsequence preferably does not occur so frequently that its presence conveys little information. For example, a probe 25 recognizing every gene conveys no information.

The optimal choice is for each target subsequence to have a probability of occurrence in all the genes or sequences that can appear in a sample or colony of approximately 50%; a preferable choice is a probability of 30 occurrence between 10 and 50%. Typically for human cDNA libraries, target subsequences of length 4 to 6 meet this condition, as longer sequences occur too infrequently to make useful hash codes. Additionally, the presence of one target subsequence is preferably independent of the presence of any 35 other target subsequence in the same sequence or gene. These two criteria ensure that a hash code for a sample, consisting of indications of which target subsequences are present, is

maximally likely to represent a unique gene or DNA sequence with minimum of wasted code words not specifying any gene. Such a hash code is an efficient representation of sequences or genes.

5 The maximal number of genes or sequences that can be represented by a hash code is 2^n , where n is the number of target subsequences. A simple test to determine whether the target subsequences occur frequently enough in the expected gene library is made by comparing the actual probabilities of 10 the two hash codes that have all target subsequences either present or absent to the ideal probabilities of these codes. If p is the probability that any target subsequence occurs in a given sequence in the library, then probability that none of the target subsequences occur in a random gene is $(1-p)^n$. 15 The closer the ratio $(1-p)^n/2^n$ is to 1 the more efficient is the code. Similarly, the closer $p^n/2^n$, the ratio of the probabilities that all the target subsequences are present to the ideal probability conveying maximum information, is to 1 the more efficient is the code. We see the optimal p is 20 close to 2^{-1} .

The preferred method of selecting target subsequences meeting the probability of occurrence and independence criteria is to use a database containing sequences generally expected to be present in the samples to 25 be analyzed, for example human GenBank sequences for human tissue derived samples. From a sequence database, oligomer frequency tables are compiled containing the frequencies of, preferably, all 4 to 8-mers. From these tables, candidate subsequences with the desired probability of occurrence are 30 selected. Each candidate target subsequence is then checked for independent occurrence, by, for example, checking that the conditional probability for a hit by any selected pair of candidates is approximately the product of the probabilities of the individual candidate hit probabilities. Candidate 35 target subsequences meeting both occurrence and independence criteria are possible target subsequences. A sufficient

number, typically 20, of any of these subsequences can be selected as target subsequences for a hash code.

Preferably, but optionally, the initially set of target subsequences can be optimized, using information on 5 the actual occurrences of the initially selected target subsequences in the sequence database, resulting in a set of target subsequences selected which recognizes a maximum number of genes with a minimum number of sequences and with a minimum amount of recognition ambiguity. Alternatively, this 10 optimization can also be performed on a sub-set of the database comprised of sequences or genes of particular biological or medical interest, for example, the set of all oncogenes or growth factors. In this manner, fewer target subsequences can be chosen which distinguish more efficiently 15 among a set of sequences or genes of particular interest and distinguish that set of genes from the sequences of the remainder of the sample.

This combinatorial optimization problem is computationally intensive to solve exactly. A number of 20 approximate techniques can be used to obtain efficient nearly optimal solutions. The preferred but not limiting technique is to use simulated annealing (Press et al., 1986, Numerical Recipes - The Art of Scientific Computing, Sec. 10.9, Cambridge University Press, Cambridge, U.K.). The 25 experimental design and optimization are described in detail in the following section.

Example 6.6 illustrates the results of the simulated annealing optimization method. Simulated annealing generally produces a choice of subsequences that achieve the 30 same resolution while using approximately 20% fewer total sequences than a selection guided only by the probability principles previously described. This level of optimization is likely to improve with larger and less redundant databases that represent longer genes.

35 An alternative to using single target subsequences is to use sets of target subsequences, recognized by sets of identically labeled hybridization probes, to generate one

presence or absence indication for the hash code. In this alternative, sets of longer target subsequences would be chosen such that the presence of any target subsequence in the set is a presence indication. Absence means no element 5 of the set is present. If the sets are chosen so that their probability of presence in a single sequence is near 50%, preferably from 10 to 50%, and the presence or absence of one set is independent of the presence or absence of any other set, such sets can be used to construct codes equally well as 10 single subsequences. A resulting code will be efficient and can be further optimized by simulated annealing, as for single target subsequence codes. Target sets of longer subsequences are preferable where experimental recognition of shorter subsequences is less specific and reproducible, as 15 for example is true where short DNA oligomers are used as hybridization probes for recognition. As a further alternative, a code can consist of presence or absence indications of mixed target sets of subsequences and single target subsequences.

20 Probes for a target subsequence are preferably PNA oligomers, or less preferably DNA oligomers, which hybridize to the subsequence of interest. Use of sets of degenerate DNA oligomers to more specifically and reliably hybridize to short DNA subsequences has been described in relation to the 25 PCR implementation of QEA™ methods. The use of PNAs is preferred in the colony calling embodiment since PNA oligomers, due to their more favorable hybridization energetics, more specifically and reliably hybridize to shorter complementary DNA subsequences than do DNA oligomers.

30 Reliable hybridization occurs for PNA 6 to 8-mers and longer. Probing shorter subsequences preferably uses fully degenerate sets of PNA oligomers, as is the case for DNA oligomers.

PNAs are even more preferable when, in the alternative, the hash code comprises presence or absence 35 indication of target sets of longer subsequences. In this case, many more DNA probes are generally required than PNA probes. As PNA 6 to 8-mers reliably hybridize, target sets

can consist of subsequences of length 6 to 8. Since DNA oligomers of this length may not reliably hybridize, each subsequence in the set must in turn be represented by a further degenerate set of DNA oligomers, requiring thereby a 5 set of sets.

The experimental method of colony calling comprises three principal steps: first, arraying cDNA libraries on filters or other suitable substrates; second, PNA hybridization and detection, alternatively DNA hybridization 10 can be used; and third, interpreting the resulting hash code to determine the sequence in the sample.

The first step, which can be omitted if arrayed cDNA libraries are already available, is constructing and arraying cDNA libraries. Any methods known in the art may be 15 used. For example, cDNA libraries from normal or diseased tissues can be constructed according to Example 6.3.

Alternatively, the human cDNA libraries constructed by M.B. Soares and colleagues are available as high density arrays on filters and can be used for the practice of this method. See 20 Soares et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:9228-32. The ability to spot up to thousands of cDNA clones or colonies on filters suitable for hybridization is an established technology. This service is now provided by several companies, including the preferred supplier Research 25 Genetics (Huntsville, AL). The protocol of Example 6.7 can be used to generate these arrays from cDNA libraries.

The second step is probe (e.g., PNA) hybridization and detection. Fluorescently labeled PNA oligomers are available from PerSeptive Biosystems (Bedford, MA) or can be 30 synthesized. PNAs are designed to be complementary to the chosen target subsequences and to have a maximum number of distinguishable labels for simultaneous hybridization with multiple oligomers. PNA hybridization is performed according to standard protocols developed by the manufacturer and 35 detailed in Example 6.7. Detection of the PNA signals uses optical spectrographic means to distinguish fluorochrome emissions similar to those used in DNA analysis instruments,

but appropriately modified to recognize spots on filters as opposed to linearly arrayed bands.

The third step, interpretation of the hash code, is done by the computer implemented method described in the 5 following section.

In an alternative embodiment, the intensity of the detected hybridization signal indicates the number of times the probe binds to the sample sequence. In this manner the 10 number of recognized target subsequences present in the sample can be determined. This information can be used to more precisely classify or identify a sample.

5.6. CC ANALYSIS AND DESIGN METHODS

The colony calling ("CC") computer implemented 15 methods are similar to QEA™ computer methods. As for QEA™, the experimental analysis methods are described before the experimental design methods.

5.6.1. CC EXPERIMENTAL ANALYSIS METHODS

20 The analysis methods make use of a mock experiment concept. First, a database is selected to represent possible sequences in the sample by the same methods as described for QEA™ analysis. These are illustrated and described with reference to Fig. 6A. For CC, an experimental definition is 25 simply a list of N_p target subsequences, where N_p is preferably between 16 and 20. Next, a mock experiment generates one hash code for each sequence in the selected database, each hash code being a string of N_p binary digits wherein the n 'th digit is a 1 (0) if the n 'th target 30 subsequence does (does not) hybridize with the sequence. The results of all the mock experiments determine the pattern of hash codes expected. This pattern is output in a code table of all possible hash codes in which, for each hash code, there is a list of all accession numbers of sequences with 35 this code.

This method is illustrated in more detail in Fig. 15. The method starts at step 1901 and at step 1902 it

inputs a selected database and on experimental definition consisting of N_p target subsequences. Step 1903 initializes a table which for each of the 2^{N_p} hash codes can contain a list of possible accession numbers which have this hash code.

5 Step 1904 is a DO loop which iterates through all sequences in the database. For a particular sequence, step 1905 checks for each target subsequence whether that subsequence hybridizes to the sequence. This is implemented by string matching in a manner similar to step 1303 of Fig. 9. A 10 binary hash code is constructed from this hybridization information, and step 1906 adds the accession number of the sequence to the list of accession numbers associated with this hash code in the code table. Step 1907 outputs the code table and the method ends at step 1908.

15 Having built a pattern of simulated hash code in a code table, analysis of an experiment requires only simple table look-up. A colony is hybridized with each of the N_p recognition means for the target subsequences. The results of the hybridization are used to construct a resulting hash 20 code. This code table for this hash code entry then contains a list of sequence accession numbers that are possible candidates for the sample sequence. If the list contains only one element, then the sample has been uniquely identified. If the list contains more than one element, the 25 identification is ambiguous. If the list is empty, the sample is not in the selected database and may possibly be a previously unknown sequence.

Alternately, as for QEA™ experimental analysis, a code table can be dispensed with if only a few hash codes 30 need to be looked up from only a few experiments. Then the DNA database is scanned sequence by sequence for those sequences generating the hash code of interest. If many hash codes from many experiments need to be analyzed, a code table is more efficient. The quantitative decision of when to 35 build a code table depends on the costs of the various operations and the size of DNA database, and can be performed

as is well known in the computer arts. Without limitation, this description is built on the use of a code table.

For those embodiments where the recognition means can each recognize a subset of target subsequences, code table construction must be modified accordingly. Such embodiments, for example, can involve DNA oligomer probes which due to their length can hybridize with an intended target subsequences and those subsequences which differ by 1 base pair from the intended target. In such embodiments, step 1905 checks whether each member of such a set of target subsequences is found in the sample sequence. If any member is found in the sequence, then this information is used to construct the hash code.

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5.6.2. CC EXPERIMENTAL DESIGN METHODS

As for QEA™, the goal of CC experimental design is to maximize the amount of information from a CC hybridization experiment. This is also performed by defining an information measure and choosing an optimization method which maximizes this measure.

The preferred information measure is the number of occupied hash codes. This is equivalent to minimizing the number of accession numbers which can result in a given hash code. In fact for N , greater than about 17 to 18, that is for 2^N greater than the number of expressed human genes (about 100,000), maximizing the number of occupied hash codes can result in each hash code representing a single sequence. Such a unique code contains the maximum amount of information. The invention is adaptable to other CC information measures. For example, if only a subset of the possible sequences are of interest, an appropriate measure would be the number of such sequences which are uniquely represented by a hash code. As for QEA™, these are sequences of interest.

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One optimization algorithm is exhaustive search. In exhaustive search, all subsequences of length less than approximately 10 are tried in all combinations in order to

find the optimum combination producing the best hash code according to the chosen information measure. This method is inefficient. The preferred algorithm for optimizing the information from an experiment is simulated annealing. This 5 is performed by the method illustrated and described with respect to Fig. 13A. For CC, the following preferred choices are made.

The energy is taken to be 1.0 divided by the information content; alternatively, any monotonically 10 decreasing function of the information content can be used. The energy is determined by performing the mock experiment of Fig. 15 using a particular experimental definition and then applying the measure to the resulting code table. For example, if the number of occupied hash codes is the 15 information measure, this number can be computed by simply scanning the code table and counting the number of table entries with non-empty accession number lists. The Boltzman constant is again taken to be 1 so that the temperature equals the energy. The initial temperature is preferably 20 1.0. The minimum energy and temperature, E_0 and T_0 , respectively, are determined by the information measure. For example, with the prior choices for energy function and information measure, E_0 , which equals T_0 , is 1.0 divided by the number of sequences in the selected database.

25 The method of generating a new experimental definition from an existing definition is to pick randomly one target subsequence and to perform one of the following moves: (1) randomly modifying one or more nucleotides; (2) adding a random nucleotide; and (3) removing a random 30 nucleotide. A modification is discarded if it results in two identical target subsequences. Further, it is desirable to discard a modification if the resulting subsequence has an extreme probability of binding to sequences in the database. For example, if the modified subsequence binds with a 35 probability less than approximately 0.1 or more than approximately 0.5 to sequences in the selected database, it should be discarded. To generate a new experiment, one of

these moves is randomly selected and carried out on the existing experimental definition. Alternatively, the various moves can be unequally weighted. The invention is further adaptable to other methods of generating new experiments.

5 Preferably, generation methods used will randomly generate all possible experiments. An initial experimental definition can be picked by taking N , randomly chosen subsequences or by using subsequences from prior optimization.

Finally, the two execution parameters defining the 10 "annealing schedule", that is the manner in which the temperature is decreased during the execution of the simulated annealing method, are defined and chosen as in QEA™. The number of iterations in an epoch, denoted by N , is preferably taken to be 100 and the temperature decay factor, 15 denoted by f , is preferably taken to be 0.95. Both N and f may be systematically varied case-by-case to achieve a better experimental definition with lower energy and a higher information measure.

With these choices the simulated annealing 20 optimization method of Fig. 13A can be performed to obtain an optimized set of target subsequences. To determine an optimum N_p , different initial N_p can be selected, the prior design optimization performed, and the results compared. The N_p with the maximum information measure is optimum for the 25 selected database.

5.6.3. CC QUANTITATIVE EMBODIMENT

To make use of quantitative detection information the pattern of simulated hash codes stored in the code table 30 is augmented with additional information. For each hash code in the table and each sequence giving rise to that hash code, this additional information comprises recording the number of times each target subsequence is found in such a sequence. These numbers are simply determined by scanning the entire 35 sequence and counting the number of occurrences of each target subsequence.

An exemplary method to perform hash code look up in this augmented table is to first find the sequences giving rise to a particular hash code as a binary number, and second to pick from these the most likely sequence as that sequence 5 having the most similar pattern of subsequence counts to the detected quantitative hybridization signal. An exemplary method to determine such similarity is to linearly normalize the detected signal so that the smallest hybridization signal is 1.0 and then to find the closest sequence by using a 10 Euclidean metric in an n-dimensional code space.

For CC experimental design, each pattern of subsequence counts may alternatively be considered as a distinct code entry for evaluation of an information measure. This is instead of considering each hash code alone a 15 distinct entry.

5.7. APPARATUS FOR PERFORMING THE METHODS OF THE INVENTION

The apparatus of this invention includes means for performing the recognition reactions of this invention in a 20 preferably automated fashion, for example by the protocols of § 6.4.3, and means for performing the computer implemented experimental analysis and design methods of this invention. Although the subsequent discussion is directed to embodiments 25 of apparatus for QEA™ embodiments of this invention, similar apparatus is adaptable to the CC embodiments. Such adaption includes using, in place of the corresponding components for QEA™ embodiments, automatic laboratory instruments appropriate for making and hybridizing arrays of clones and for reading the results of the hybridizations, and using 30 programs implementing the computer analysis and design methods for the CC embodiments described in Sec. 5.6.

Fig. 12A illustrates an exemplary apparatus for QEA™ embodiments of this invention, and with the described adaption, also for the CC embodiments of this invention. 35 Computer 1601 can be, alternatively, a UNIX based work station type computer, an MS-DOS or Windows based personal computer, a Macintosh personal computer, or another

equivalent computer. In a preferred embodiment, computer 1601 is a PowerPC™ based Macintosh computer with software systems capable of running both Macintosh and MS-DOS/Windows programs.

5 Fig. 12B illustrates the general software structure in RAM memory 1650 of computer 1601 in a preferred embodiment. At the lowest software level is Macintosh operating system 1655. This system contains features 1656 and 1657 for permitting execution of UNIX programs and MS-DOS 10 or Windows programs alongside Macintosh programs in computer 1601. At the next higher software level are the preferred languages in which the computer methods of this invention are implemented. LabView 1658, from National Instruments (Dallas, TX), is preferred for implementing control routines 15 1661 for the laboratory instruments, exemplified by 1651 and 1652, which perform the recognition reactions and fragment separation and detection. C or C++ languages 1659 are preferred for implementing experimental routines 1662, which are described in Sec. 5.4 and 5.6. Less preferred, but 20 useful for rapid prototyping, are various scripting languages known in the art. PowerBuilder 1660, from Sybase (Denver, CO), is preferred for implementing the user interfaces to the computer implemented routines and methods. Finally, at the highest software level are the programs implementing the 25 described computer methods. These programs are divided into instrument control routines 1661 and experimental analysis and design routines 1662. Control routines 1661 interact with laboratory instruments, exemplified by 1651 and 1652, which physically perform QEA™ and CC protocols. Experimental 30 routines 1662 interact with storage devices, exemplified by devices 1654 and 1653, which store DNA sequence databases and experimental results.

Returning to Fig. 12A, although only one processor is illustrated, alternatively, the computer methods and 35 instrument control interface can be performed on a multiprocessor or on several separate but linked processors, such that instrument control methods 1661, computational

experimental methods 1661, and the graphical interface methods can be on different processors in any combination or sub-combination.

Input/output devices include color display device 5 1620 controlled by a keyboard and standard mouse 1603 for output display of instrument control information and experimental results and input of user requests and commands. Input and output data are preferably stored on disk devices such as 1604, 1605, 1624, and 1625 connected to computer 1601 10 through links 1606. The data can be stored on any combination of disk devices as is convenient. Thereby, links 1606 can be either local attachments, whereby all the disks can be in the computer cabinet(s), LAN attachments, whereby the data can be on other local server computers, or remote 15 links, whereby the data can be on distant servers.

Instruments 1630 and 1631 exemplify laboratory devices for performing, in a partly or wholly automatic manner, QEA™ recognition reactions. These instruments can be, for example, automatic thermal cyclers, laboratory 20 robots, and controllable separation and detection apparatus, such as is found in the applicants' copending U.S. Patent Application 08/438,231 filed May 9, 1995. Links 1632 exemplify control and data links between computer 1601 and controlled devices 1631 and 1632. They can be special buses, 25 standard LANs, or any suitable link known in the art. These links can alternatively be computer readable medium or even manual input exchanged between the instruments and computer 1601. Outline arrows 1634 and 1635 exemplify the physical flow of samples through the apparatus for performing 30 experiments 1607 and 1613. Sample flow can be either automatic, manual, or any combination as appropriate. In alternative embodiments there may be fewer or more laboratory devices, as dictated by the current state of the laboratory automation art.

35 On this complete apparatus, a QEA™ experiment is designed, performed, and analyzed, preferably in a manner as automatic as possible. First, a QEA™ experiment is designed,

according to the methods specified in Sec. 5.4.2 as implemented by experimental routines 1662 on computer 1601. Input to the design routines are databases of DNA sequences, which are typically representative selected database 1605 5 obtained by selection from input comprehensive sequence database 1604, as described in Sec. 5.4.1. Alternatively, comprehensive DNA databases 1604 can be used as input. Database 1604 can be local to or remote from computer 1601. Database selection performed by processor 1601 executing the 10 described methods generates one or more representative selected databases 1605. Output from the experimental design methods are tables, exemplified by 1609 and 1615, which, for a QEA™ RE embodiment, specify the recognition reaction and the REs used for each recognition reaction.

15 Second, the apparatus performs the designed experiment. Exemplary experiment 1607 is defined by tissue sample 1608, which may be normal or diseased, experimental definition 1609, and physical recognition reactions 1610 as defined by 1609. Where instrument 1630 is a laboratory robot 20 for automating reaction, computer 1601 commands and controls robot 1630 to perform reactions 1610 on cDNA samples prepared from tissue 1608. Where instrument 1631 is a separation and detection instrument, the results of these reactions are then transferred, automatically or manually, to 1631 for 25 separation and detection. Computer 1601 commands and controls performance of the separation and receives detection information. The detection information is input to computer 1601 over links 1632 and is stored on storage device 1624, along with the experimental design tables and information on 30 the tissue sample source for processing. Since this experiment uses, for example, fluorescent labels, detection results are stored as fluorescent traces 1611.

Experiment 1613 is processed similarly along sample pathway 1633, with robot 1630 performing recognition 35 reactions 1616 on cDNA from tissue 1608 as defined by definition 1615, and device 1631 performing fragment separation and detection. Fragment detection data is input

by computer 1601 and stored on storage device 1625. In this case, for example, silver staining is used, and detection data is image 1617 of the stained bands.

During experimental performance, instrument control 5 routines 1661 provide the detailed control signals needed by instruments 1630 and 1631. These routines also allow operator monitoring and control by displaying the progress of the experiment in process, instrument status, instrument exceptions or malfunctions, and such other data that can be 10 of use to a laboratory operator.

Third, interactive experimental analysis is performed using the database of simulated signals generated by analysis and design routines 1662 as described in Sec.

5.4.2 and 5.4.3. Simulated database 1612 for experiment 1607 15 is generated by the analysis methods executing on processor 1601 using as input the appropriate selected database 1605 and experimental definition 1609, and is output in table 1612. Similarly table 1618 is the corresponding simulated database of signals for experiment 1613, and is generated 20 from appropriate selected database 1605 and experimental definition 1615. A signal is made unambiguous by experimental routines 1662 that implement the methods described in Sec. 5.4.3.

Display device 1602 presents an exemplary user 25 interface for the data generated by the methods of this invention. This user interface is programmed preferably by using the Powerbuilder display front end. At 1620 are selection buttons which can be used to select the particular experiment and the particular reaction of the experiment 30 whose results are to be displayed. Once the experiment is selected, histological images of the tissue source of the sample are presented for selection and display in window 1621. These images are typically observed, digitized, and stored on computer 1601 as part of sample preparation. The 35 results of the selected reaction of the selected experiment are displayed in window 1622. Here, a fluorescent trace output of a particular labeling is made available. Window

1622 is indexed by marks 1626 representing the possible locations of DNA fragments of successive integer lengths.

Window 1623 displays contents from simulated database 1612. Using, for example, mouse 1603, a particular 5 fragment length index 1626 is selected. The processor then retrieves from the simulated database the list of accession numbers that could generate a peak of that length with the displayed end labeling. This window can also contain further information about these sequences, such as gene name, 10 bibliographic data, etc. This further information may be available in selected databases 1605 or may require queries to the complete sequence database 1604 based on the accession numbers. In this manner, a user can interactively inquire into the possible sequences causing particular results and 15 can then scan to other reactions of the experiment by using buttons 1620 to seek other evidence of the presence of these sequences.

It is apparent that this interactive interface has further alternative embodiments specialized for classes of 20 users of differing interests and goals. For a user interested in determining tissue gene expression, in one alternative, a particular accession number is selected from window 1623 with mouse 1603, and processor 1601 scans the simulated database for all other fragment lengths and their 25 recognition reactions that could be produced by this accession number. In a further window, these lengths and reactions are displayed, and the user allowed to select further reactions for display in order to confirm or refute the presence of this accession number in the tissue sample. 30 If one of these other fragments are generated uniquely by this sequence (a "good sequence", see *supra*), that fragment can be highlighted as of particular interest. By displaying the results of the generating reaction of that unique fragment, a user can quickly and unambiguously determine 35 whether or not that particular accession number is actually present in the sample.

In another interface alternative, the system displays two experiments side by side, displaying two histological images 1621 and two experimental results 1622. This allows the user to determine by inspection signals 5 present in one sample and not present in the other. If the two samples were diseased and normal specimens of the same tissue, such signals would be of considerable interest as perhaps reflecting differences due to the pathological process. Having a signal of interest, preferably repeatable 10 and reproducible, a user can then determine the likely accession numbers causing it by invoking the previously described interface facilities. In a further elaboration of this embodiment, system 1601 can aid the determination of signals of interest by automating the visual comparison by 15 performing statistical analysis of signals from samples of the same tissue in different states. First, signals reproducibly present in tissue samples in the same state are determined, and second, differences in these reproducible signals across samples from the several states are compared. 20 Display 1602 then shows which reproducible signals vary across the states, thereby guiding the user in the selection of signals of interest.

The apparatus of this invention has been described above in an embodiment adapted to a single site 25 implementation, where the various devices are substantially local to computer 1601 of Fig. 12A, although the various links shown could also represent remote attachments. An alternative, explicitly distributed embodiment of this apparatus is illustrated in Fig. 12C. Shown here are 30 laboratory instruments 1670, DNA sequence database systems 1684, and computer systems 1671 and 1673, all of which cooperate to perform the methods of this invention as described above.

These systems are interconnected by communication 35 medium 1674 and its local attachments 1675, 1676, and 1677 to the various systems. This medium may be any dedicated or shared or local or remote communication medium known in the

art. For example, it can be a "campus" LAN network extending perhaps a few kilometers, a dedicated wide area communication system, or a shared network, such as the Internet. The system local attachments are adapted to the nature of medium 5 1674.

Laboratory instruments 1670 are commanded by computer system 1671 to perform the automatable steps of the recognition reactions, separation of the reaction results, and detection and transmission of resulting signals through 10 link 1672. Link 1672 can be any local or remote link known in the art that is adapted to instrument control, and may even be routed through communication medium 1674..

DNA sequence database systems 1684 with various sequence databases 1685 may be remote from the other systems, 15 for example, by being directly accessed at their sites of origin, such as Genbank at Bethesda, MD. Alternatively, parts or all of these databases may be periodically downloaded for local access by computer systems 1671 and 1672 onto such storage devices as discs or CD-ROMs.

20 Computer system 1671, including computer 1681, storage 1682, and display 1633, can perform various methods of this invention. For example, it can perform solely the control routine for control and monitoring of instrument system 1670, whereby experimental design and analysis are 25 performed elsewhere, as at computer system 1673. In this case, system 1671 it would typically be operated by laboratory technicians. Alternatively, system 1671 can also perform experimental designs, which meet the requirements of remote users of sample analysis information. In another 30 embodiment, system 1671 can carry out all the computer implemented methods of this invention, including final data display, in which case it would be operated by the final users of the analysis information.

Computer system 1673, including computer 1678, 35 storage 1679, and display 1680, can perform a corresponding range of functions. However, typically system 1673 is remotely located and would be used by final users of the DNA

sample information. Such users can include clinicians seeking information to make a diagnosis, grade or stage a disease, or guide therapy. Other users can include pharmacologists seeking information useful for the design or 5 improvement of drugs. Finally, other users can include researchers seeking information useful to basic studies in cell biology, developmental biology, etc. It is also possible that a plurality of computer systems 1673 can be linked to laboratory system 1670 and control system 1671 in 10 order to provide for the analysis needs of a plurality of classes of users by designing and causing the performance of appropriate experiments.

It will be readily apparent to those of skill in the computer arts that alternative distributed embodiments of 15 the apparatus of this invention, along with alternative functional allocations of the computer implemented methods to the various distributed systems, are equally possible.

All the computer implemented methods of this invention can be recorded for storage and transport on any 20 computer readable memory devices known in the art. For example, these include, but are not limited to, semiconductor memories - such as ROMs, PROMs, EPROMs, EEPROMS, etc. of whatever technology or configuration - magnetic memories - such as tapes, cards, disks, etc of whatever density or size 25 - optical memories - such as optical read-only memories, CD-ROM, or optical writeable memories - and any other computer readable memory technologies.

Also, although this apparatus has been described primarily with reference to QEA™ analysis of human tissue 30 samples, the laboratory instruments and associated control, design, and analysis computer systems are not so limited. They are also adaptable to performing the CC embodiment of this invention and to the analysis of other samples, such as from animal models or in vitro cultures.

35 The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

6. EXAMPLES

6.1. SUBSEQUENCE HIT AND LENGTH INFORMATION

This example illustrates QEA™ signals generated by 5 a PCR embodiment. From the October 1994 GenBank database, 12,000 human first continuous coding domain sequences ("CDS") were selected. This selection resulted in a selected database of sequences with a bias toward shorter genes, the average length of the selected CDSs being 1000 bp instead of 10 the typical coding sequence length of 1800-2000 bp, and with no guarantee that sequences were not be repeated in the selected database. From this database, tables containing the probability of occurrence of all 4 to 6-mer sequences were constructed.

15 Then Eqns. 1 and 2 were solved for $N = 12,000$ and $L = 1,000$ resulting in $p = 0.17$ and $M = 108$. Five 6-mer target subsequences with this probability of occurrence were chosen from the 6-mer tables and grouped into four pairs: CAGATA-TCTCAC, CAGATA-GGTCTG, CAGATA-GCTCAA CAGATA-CACACC. Analyses 20 comprising mock digestions (see Sec. 5.4.1) of the selected database of CDSs were then performed for these four pairs of target subsequences.

The histogram of Fig. 1 presents the results of these analyses. Along axis 102 is the length of fragments, 25 as would be observed in a gel separation of the amplified fragments of a QEA™ reaction recognizing these target subsequences. Along axis 101 is the number of fragments at a given length. For example, spike 103 at a length of approximately 800 base pairs represents three fragments of 30 the same length. Multiple fragments at one length may occur either because several CDSs have one target subsequence pair spaced this length, because one CDS has several target subsequence pairs spaced this length, because of redundancy in the selected CDSs, or because signals of this length were 35 generated by more than one pair of target subsequences. Spike 104 at a slightly longer length represents a single fragments. This fragment is generated from a unique sequence

and provides a unique indication of its presence in a cDNA mixture, that is, this is a good sequence.

6.2. RESTRICTION ENDONUCLEASES

5 Tables 1-4 list all palindromic 4-mer and 6-mer potential RE recognition sequences. RE enzymes recognizing each site, where known, are also listed, along with an exemplary commercial supplier. Over 85% of possible sequences spanning a wide range of occurrence probabilities 10 have a known RE recognizing and cutting within the sequence.

The frequency of these sequences was determined, as in example 6.1, in 12,000 human first continuous coding domain sequences selected from the October 1994 GenBank database. The tables are sorted in order of increasing 15 recognition occurrence probability. The bar in the recognition sequence indicates the site in the recognition sequence where the RE cuts.

The following vendor abbreviations are used: New England Biolabs (Beverly, MA) ("NEB"), Stratagene (La Jolla, CA), 20 Boehringer Mannheim (Indianapolis, IN) ("BM"), and Gibco BRL division of Life Technologies (Gaithersburg, MD) ("BRL").

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TABLE 1: THE 4-MER RESTRICTION SITES

Recognition Sequence	CDS Frequency	RE	Overhang	Vendor
C GCG	0.36	SelI	2	
C TAG	0.44	MaeI	2	NEB
T TAA	0.45	MseI	2	NEB
TATA	0.45			
GCG C	0.50	HhaI	2	NEB
ATAT	0.50			
A CGT	0.52	MaeII	2	BM
T CGA	0.53	TaqI	2	NEB
AATT	0.53	Tsp5G91	4	NEB
C CGG	0.61	MspI	2	NEB
G TAC	0.64	Csp6I	2	NEB
GATC	0.67	Sau3AI	4	NEB
CATG	0.68	NlaIII	4	NEB
TG CA	0.78	CviRI	0	
AG CT	0.78	AluI	0	NEB
GG CC	0.79	HaeIII	0	NEB

25

30

35

TABLE 2: THE FIRST 20 6-MER RESTRICTION SITES

	Sequence	CDS Frequency	RE	Overhang	Vendor
5	TCG CGA	0.01	NruI	0	NEB
	TAC GTA	0.02	SnaBI	0	NEB
	C GTACG	0.02	BsiWI	4	NEB
	CGAT CG	0.02	PvuI	2	NEB
	A CGCGT	0.03	MluI	4	NEB
10	A CTAGT	0.03	SpeI	4	NEB
	G TCGAC	0.04	SalI	4	NEB
	AA CGTT	0.04	Psp1406I	2	NEB
	A CCGGT	0.04	AgeI	4	NEB
	G CTAGC	0.04	NheI	4	NEB
15	TATATA	0.04			
	GTT AAC	0.05	HpaI	0	NEB
	TAGCTA	0.05			
	TAATTA	0.05			
	GTA TAC	0.05	Bst1107I	0	NEB
20	CTATAG	0.05			
	CGCGCG	0.05			
	C CTAGG	0.06	AvrII	4	NEB
	TT CGAA	0.06	SfaI	2	BM
	AT CGAT	0.06	ClaI	2	NEB

TABLE 3: THE MIDDLE 20 6-MER RESTRICTION SITES

Sequence	CDS Frequency	RE	Overhang	Vendor
5	C TTAAG	AlflII	4	NEB
	T CTAGA	XbaI	4	NEB
	ATATAT			
	AT TAAT	VspI	2	BRL
	G CGCGC	BssHII	4	NEB
10	C AATTG	MunI	4	NEB
	GACGT C	AatII	4	NEB
	TTATAA			
	TGC GCA	FspI	0	NEB
	C TCGAG	XbaI	4	NEB
15	GAT ATC	EcoRV	0	NEB
	CA TATG	NdeI	2	NEB
	ATGCA T	NsiI	4	NEB
	AGC GCT	Ecc47III	0	NEB
	AAT ATT	SspI	0	NEB
20	T CCGGA	AccIII	4	Stratagene
	TTT AAA	DraI	0	NEB
	A CATGT	BspLVII	4	
	CAC GTG	Eco72I	0	Stratagene
	CCGC GG	SacII	2	NEB

TABLE 4: THE LAST 24 6-MER RESTRICTION SITES

Sequence	CDS Frequency	RE	Overhang	Vendor
GCATG C	0.13	SphI	4	NEB
TTGCAA	0.13			
A AGCTT	0.13	HindIII	4	NEB
G TGCAC	0.13	ApaLI	4	NEB
AAATTT	0.14			
AGT ACT	0.15	ScaI	0	NEB
G AATTC	0.15	EcoRI	4	NEB
GGTAC C	0.15	KpnI	4	NEB
T GTACA	0.15	Bsp1407I	4	NEB
C GGCCG	0.15	EagI	4	NEB
G CCGGC	0.16	NgoMI	4	NEB
GGC GCC	0.16	NarI	0	NEB
T GATCA	0.16	BclI	4	NEB
T CATGA	0.17	BspHI	4	NEB
C CCGGG	0.19	SmaI	4	NEB
G GATCC	0.19	BamHI	4	NEB
A GATCT	0.20	BglII	4	NEB
AGG CCT	0.22	StuI	0	NEB
GGGCC C	0.24	ApaI	4	NEB
C CATGG	0.24	NcoI	4	NEB
GAGCT C	0.25	SacI	4	NEB
TGG CCA	0.33	MscI	0	NEB
CAG CTG	0.42	PvuII	0	NEB
CTGCA G	0.43	PstI	4	NEB

6.3. RNA EXTRACTION AND cDNA SYNTHESIS

These protocols describe preferred methods for extraction of RNA from tissue samples and for synthesis of de-phosphorylated cDNA from the extracted RNA.

5

6.3.1. RNA EXTRACTION

In general, RNA extraction is done using Triazol reagent from Life Technologies (Gaithersburg, MD) following the protocol of Chomczynski et. al., 1987, *Annal. Biochem.*

10 162:156-59 and Chomczynski et. al., 1993, *Biotechniques* 15:532-34,536-37. Total RNA is first extracted from tissues, treated with RNase-free DNase I from Pharmacia Biotech (Uppsala, Sweden) to remove contaminating genomic DNA, followed by messenger RNA purification using oligo (dT)

15 magnetic beads from Dynal Corporation (Oslo, Norway), and then used for cDNA synthesis.

If desired, total cellular RNA can be separated into sub-pools prior to cDNA synthesis. For example, a super-pool of endoplasmic reticulum associated RNA is enriched for 20 RNA producing proteins having an extra-cellular or receptor function.

In more detail, the following protocol is preferred for RNA extraction from tissue samples.

25 Tissue Homogenization and Total RNA Extraction:

A voxel is used to describe the specific piece of tissue to be analyzed. Most frequently it will refer to grid punches corresponding to pathologically characterized tissue sections.

30 1. It is important that tissue voxels be quick frozen in liquid nitrogen immediately after dissection, and stored at -70°C until processed.

2. The weight of the frozen tissue voxel is measured and recorded.

35 3. Tissue voxels are pulverized and ground in liquid nitrogen, either with a porcelain mortar and pestle, or by stainless steel pulverizers, or alternative means. This

tissue is ground to a fine powder and is kept on liquid nitrogen.

4. The tissue powder is transferred to a tube containing Triazol reagent (Life Technologies, Gaithersburg, MD) with 1 5 ml of reagent per 100 mg of tissue and is dispersed in the Triazol using a Polytron homogenizer from Brinkman Instruments (Westbury, NY). For small tissue voxels less than 100 mg, a minimum of 1 ml of Triazol reagent should be used for efficient homogenization.

10 5. Add 0.1 volumes BCP (1-bromo-3-chloropropane) (Molecular Research, Cincinnati, OH) and mix by vortexing for 30 seconds. Let the mixture stand at room temperature for 15 minutes.

6. Centrifuge for 15 minutes at 4°C at 12,000X G.

15 7. Remove the aqueous phase to a fresh tube and add 0.5 volumes isopropanol per original amount of Triazol reagent used and mix by vortexing for 10 seconds. Let the mixture stand at room temperature for 10 minutes.

8. Centrifuge at room temperature for 10 minutes at 12,000X 20 G.

9. Wash with 70% ethanol and centrifuge at room temperature for 5 minutes at 12,000X G.

10. Remove the supernatant and let the centrifuge tube stand to dry in an inverted position.

25 11. Resuspend the RNA pellet in water (1 µl per mg of original tissue weight) and heat to 55°C until completely dissolved.

DNase treatment:

30 1. Add 0.2 volume of 5X reverse transcriptase buffer (Life Technologies, Gaithersburg, MD), 0.1 volumes of 0.1 M DTT, and 5 units RNAGuard per 100 mg starting tissue from Pharmacia Biotech (Uppsala, Sweden).

2. Add 1 unit RNase-free DNase I, Pharmacia Biotech, per 35 100 mg starting tissue. Incubate at 37°C for 20 minutes.

The following additional steps are optional,

Opt 1. Repeat RNA extraction by adding 10 volumes of Triazol reagent.

Opt 2. Repeat steps 5 through 11.

5 3. Quantify the total RNA (from the RNA concentration obtained by measuring OD₂₆₀ of a 100 fold dilution). Store at -20°C.

Isolation of Poly A⁺ Messenger RNA:

10 Poly-adenylated mRNA is isolated from total RNA preparations using magnetic bead mediated oligo-dT detection. Kits that can be used include Dynabeads mRNA Direct Kit from Dynal (Oslo, Norway) or MPG Direct mRNA Purification Kit from CPG (Lincoln Park, NJ). Protocols are used as directed by 15 the manufacturer.

Less preferably, the following procedure can be used. The Dynal oligo(dT) magnetic beads have a capacity of 1 ug poly(A⁺) per 100 ug of beads (1 mg/ml concentration), assuming 2% of the total RNA has poly(A⁺) tails.

20

1. Add 5 volumes of Lysis/Binding buffer (Dynal) and sufficient beads to bind the estimated poly(A⁺) RNA.
2. Incubate at 65°C for 2 minutes, then at room temperature for 5 minutes.

25 3. Wash beads with 1 ml Washing buffer/LiDS (Dynal)

4. Wash beads with 1 ml Washing buffer (Dynal) 2 times.
5. Elute poly(A⁺) RNA with 1 μ l water/ug beads 2 times.

For both methods, the poly-adenylated RNA is 30 harvested in a small volume of water, quantified as above, and stored at -20°C. Typical yields of poly-adenylated RNA range from 1% to 4% of the input total RNA.

6.3.2. cDNA SYNTHESIS

35 This protocol for the synthesis of de-phosphorylated cDNA from poly (A)⁺ RNA is preferred when the

quantities of input RNA are approximately 1 μ g, or at least 200 ng or greater.

Reagents Used:

- 5 • Random Hexamers (50 ng/ μ l)
- 5X First strand buffer (BRL)
- 10 mM dNTP mix
- 100 mM DTT
- SuperScript II reverse transcriptase (BRL) (200 U/ μ l)
- 10 • *E. coli* DNA ligase (BRL) 10 U/ μ l
- *E. coli* DNA polymerase (BRL) 10 U/ μ l
- T4 DNA polymerase 2.5 U/ μ l
- *E. coli* RNaseH (BRL) 3.5 U/ μ l
- 15 • Arctic Shrimp Alkaline Phosphatase, (SAP; USB), and 10X SAP buffer (USB)
- 5X Second strand buffer (BRL)
- 3 M Na-Acetate
- Phenol:Chloroform (phenol:chloroform:isoamyl alcohol 25:24:1)
- 20 • Chloroform isoamyl alcohol (24:1)
- Absolute and 75% ethanol
- 20 ug/ μ l glycogen (BM)

25 cDNA Synthesis Protocol:

1. Mix .25-1.0 ug of poly A+ RNA with 50 ng of random hexamers in 10 μ l of water. Heat the mixture to 70°C for 10 min. and quick chill in ice-water slurry. Keep on ice for 1-2 min. Spin in microfuge for 10 secs. to collect condensate.
- 30 2. Prepare first stand reaction mix with 4 μ l 5x First strand buffer, 2 μ l 100 mM DTT, 1 μ l 10 mM dNTP mix, and 2 μ l water. Add this mix to the primer-annealed RNA from step 1. Place mixture at 37°C for 2 mins. Add 1 μ l of Superscript II (BRL) (following manufacturer's recommendations). Incubate at 37°C for 1 hr.

3. Place tubes on ice, add 30 μ l of 5x Second strand buffer, 90 μ l of cold water, 3 μ l of 10 mM dNTP, 1 μ L (10 units) of *E. coli* DNA ligase, 4 μ l (40 units) of *E. coli* DNA polymerase, and 1 μ l (3.5 units) of *E. coli* RnaseH. Incubate for 2h. at 16°C.
4. Add 2 μ l of T4 DNA polymerase (5 units) and incubate at 16°C for 5 min.
5. Add 20 μ l 10x SAP buffer, 25 μ l of water, and 5 μ l (5 units) of SAP. Incubate at 37°C for 30 min.
6. Extract cDNA with phenol-chloroform, chloroform-isoamyl alcohol. To the aqueous layer add Na-acetate to 0.3 M, 20 ug glycogen, and 2 vol of ethanol. Incubate at -20°C for 10 min., spin at 14,000 g for 10 min. Wash pellet with 75% ethanol. Dissolve pellet in 50 μ l TE.
7. Estimate the yield of cDNA using fluorometer.
8. For subsequent QEA™ processing, transfer 75 ng cDNA to a separate tube, add TE to make the concentration 600 ng/ml and put that tube in the specified box at -20°C. For storage, add Na-acetate to 0.3M and 2 vol of ethanol to the rest of cDNA and store at -80 °C.

Alternative primers for first strand synthesis known in the art can also be used for first strand synthesis. Such primers include oligo(dT) primers, phasing primers, etc.

25

6.3.3. cDNA SYNTHESIS FOR SMALL QUANTITIES OF RNA

The cDNA synthesis protocol previously described is based primarily on the method of Gubler and Hoffman (Gubler et al., 1983, "A simple and very efficient method for generating cDNA libraries," *Gene* 25:263-9) and is robust and well-proven for quantities of RNA in the 1 μ g range (200 ng and up). A more preferred protocol for RNA quantities below 200 ng takes advantage of the 5' CAP structure of RNAs (Edery et al., 1995, "An efficient strategy to isolate full-length cDNAs based on an mRNA cap retention procedure (CAPTURE)," *Mol. Cell Biol.* 15:3363-71; Kato et al., 1994, "Construction

of a human full-length cDNA bank," *Gene* 150:243-50). This protocol has a number of advantages including:

- broad scalability of RNA input quantities, making them ideal for biopsies and for other small and variable sized samples;
- capability of doing a pre-QEA™ amplification of the cDNA when very small amounts of cDNA are available;
- cDNA synthesis biased toward full-length RNAs.
- capability of introducing specific primer sites at both ends of the full-length cDNAs;
- option to eliminate the poly (A)+ RNA purification step and use total RNA.

cDNA Synthesis Protocol

- 15 1. The poly (A)†, or total, RNA (10 µg) is dephosphorylated with bacterial alkaline phosphatase (20 µl rxn; 100 mM Tris-HCl pH 7.5, 2 mM DTT; 0.2 U bacterial alkaline phosphatase, 20 U RNase inhibitor; 37°C for 30 minutes).
2. After phenol extraction and ethanol precipitation, the RNA is treated with tobacco acid pyrophosphatase. (20 µl rxn; 50 mM Na-OAc pH 6.0, 1 mM EDTA, 2 mM DTT; 0.1 U tobacco acid pyrophosphatase, 20 U RNase inhibitor; 37°C for 30 minutes).
3. Phenol extract and ethanol precipitate the decapped RNA.
- 25 4. The following DNA-RNA primer named MA24R (3 nm) is ligated to the 5-prime end using T4 RNA Ligase (20 µl rxn; Tris-HCl pH 7.5, 5 mM MgCl₂, 0.5 mM ATP, 2 mM DTT, 25% ethylene glycol; 100 U T4 RNA Ligase, 20 U RNase inhibitor; 20°C for 12 hours):
30 MA24R: dCdAdGdTdAdGdCdGdAdTdTdGdCdCdGdCdGdTdCdAdGdGdTGGA
(SEQ ID NO:??)
4. First strand synthesis is performed identically to steps 1 and 2 of the protocol previously described in Sec. 6.3.2 except that the following biotinylated primers are used to prime the cDNA:

MBTA: CGGTGGGTTGCCGTAGTCGGAT(T)₂A
(SEQ ID NO:??)

MBTC: CGGTGGGTTGCCGTAGTAGCGGAT (T)₂₅C

(SEQ ID NO:??)

MBTG: CGGTGGGTTGCCGTAGTAGCGGAT (T)₂₅G

(SEQ ID NO:??)

5 These reactions can occur in separate tubes or in one tube. The phasing effect of doing the reaction in separate tubes has the advantage of dividing the cDNA into three separate pools. 0.2 μ g of each primer is used in the reaction.

10 5. Second strand synthesis is performed identically to steps 3 and 4 of the protocol previously described in Sec. 6.3.2 using a DNA-only version of the DNA-RNA chimera is used to prime synthesis:

MA24: CAGTAGCGATTGCCGCCGTCAGGT

15 (SEQ ID NO:??)

Because the primers at both 5' ends lack phosphate groups, dephosphorylation of the resulting cDNA, e.g., by shrimp alkaline phosphatase, is no longer necessary.

6. In cases where exceedingly small amounts of cDNA are 20 synthesized (1-10 ng yields), the sample can be amplified using the following primer pair:

MA24: CAGTAGCGATTGCCGCCGTCAGGT

(SEQ ID NO:??)

MB24: CGGTGGGTTGCCGTAGTAGCGGAT

25 (SEQ ID NO:??)

For 1 ng quantities, 500-fold amplification by 8 to 10 PCR cycles (96°C 30 seconds, 57°C 1 minute, 72°C 3 minutes) provides adequate cDNA for comprehensive analysis.

30

6.3.4. ALTERNATIVE cDNA SYNTHESIS

cDNA synthesis

Alternately, cDNA can be synthesized using the Superscript™ Choice system from Life Technologies, Inc.

35 (Gaithersburg, MD). If tissue voxels are the source for the RNA, the polyadenylated RNA is not quantified, and the entire yield of polyadenylated RNA is concentrated by precipitation

with ethanol. The polyadenylated RNA is resuspended in 10 μ l of water, and 5 to 10 μ l are used for cDNA synthesis. The manufacturer's protocols are followed for RNA amounts of less than 1 μ g, using 100 ng of random hexamers are used as 5 primers. If greater than 1 μ g of polyadenylated RNA is used, the manufacturer's protocols are followed, using 50 ng of random hexamer primers per microgram of polyadenylated RNA. The resulting volume of the cDNA solution is 150 μ l. If the amount is not quantified, QEA™ test reactions can be run 10 using 1 μ l or 0.1 μ l of cDNA solution in order to determine the appropriate amount of cDNA to use for subsequent QEA™ reactions.

cDNA De-phosphorylation

15 Where cDNA is synthesized with terminal phosphates, they are preferably removed before the RE/Ligase reactions. Terminal phosphate removal from cDNA is illustrated with the use of Barents sea shrimp alkaline phosphatase ("SAP") (U.S. Biochemical Corp.) and 2.5 μ g of cDNA. Substantially less 20 (<10 ng) or more (>20 μ g) of cDNA can be prepared at a time with proportionally adjusted amounts of enzymes. Volumes are maintained to preserve ease of handling. The quantities necessary are consistent with using the method to analyze small tissue samples from normal or diseased specimens.

25

1. Mix the following reagents
 - 2.5 μ l 200 mM Tris-HCL
 - 23 μ l cDNA
 - 2 μ l 2 units/ μ l Shrimp alkaline phosphatase
- 30 The final resulting cDNA concentration is 100 ng/ μ l.
2. Incubate at 37°C for 1 hour
3. Incubate at 80°C 15 minutes to inactivate the SAP.

6.4. QEA™ PREFERRED RE METHOD

35 Protocols for the RE embodiment are designed to minimize the number of individual manipulations down, and thereby to maximize the reproducibility of QEA™ procedures.

In preferred protocols, no buffer changes, precipitations, or organic (phenol/chloroform) extractions are used, all of which lower the overall efficiency of the process and reduce its utility for general use and more specifically for its use 5 in automated or robotic procedures.

Once the cDNA has been prepared, including terminal phosphate removal, it is separated into batches of from 1 ng to at least 50 ng each and of a number equal to the desired number of individual samples that need to be analyzed. For 10 example, if six RE/ligase reactions and six analyses are needed to generate all necessary signals, six batches are made. Advantageously, QEA™ reactions can be duplicated or triplicated in order to increase precision of and confidence in the results.

15 RE/ligase reactions are performed as digestions by, preferably, a pair of REs; alternatively, one or three or more REs can be used provided that the four base pair overhangs generated by each RE differ and can each be ligated to a uniquely adapter and that a sufficiently resolved length 20 distribution results. The preferably amount of RE enzyme specified in the protocols is sufficient for complete digestion while minimizing any other exo- or endo-nuclease activity that may be present in the enzyme. Preferred and alternate RE combinations can be found in Tables 11 to 14.

25 Adapters are chosen that are unique to each RE in a reaction. They are comprised of a linker complementary to each unique RE sticky overhang and a primer which uniquely hybridizes with that linker. The hybridized primer/linker combination is called an adapter.

30 The primer/linker combination for a given RE are chosen according to the several embodiments of QEA™ reactions selected. Generally, sample primer/linker combinations are chosen according to the combinations illustrated in Table 10 for any particular RE. The primers can be labeled when the 35 detection means so require. Where one or more, or preferably all, primers have label moieties, these moieties are preferably distinguishable and can be advantageously chosen

from the fluorescent labels described in Sec. 6.11. In a QEA™ embodiment using post-PCR cleanup, one primer has a capture moiety, e.g., biotin. The capture moiety is preferably bound to one of the R-series primers, RA24 or 5 RC24, and the other primer is preferably labeled. Pairs of labeled and biotinylated primers are preferably chosen according to table 11 for the RE pairs therein listed. Finally, in the case of an SEQ-QEA™ embodiment, primers and linkers are preferably chosen according to Sec. 6.10.1.

10

6.4.1. PREFERRED RE/LIGASE & AMPLIFICATION REACTIONS

This section describes the preferred protocol for performing the RE/ligase and PCR amplification reactions with a minimum of intervention.

15

Primer-excess Adapter Set Annealing

In the preferred protocol, a primer/linker combination in the form of an adapter set specific for each RE is chosen as above. The adapter set comprises sufficient 20 adapters, hybridized primer/linker, for the RE/ligase reaction and also sufficient excess primers for the subsequent PCR amplification. Accordingly, primers do not have to be separately added to the PCR reaction mix. Adapter sets are constructed from linkers and primers according to 25 the following protocol:

1. Add to water linker and primer in a 1:20 concentration ratio (12-mer : 24-mer) with the primer at a total concentration of 50 pm per μ l.
- 30 2. Incubate at 50°C for 10 minutes.
3. Cool slowly to room temperature and store at -20°C.

RE/ligase & Amplification Protocol

35 1. Combine the following components for the QPCR mix as shown:

Reagent	Concentration	1 rxn	96 rxns
10X TB 2.0	500 mM Tris pH 9.15, 160 mM (NH ₄) ₂ SO ₄ , 20 mM MgCl ₂	5 μ l	525 μ l
5 dNTP (equimolar mixture)	10 mM	2 μ l	210 μ l
Klentaq:PFU (16:1)	25 U/ml	0.25 μ l	26.25 μ l
10 water		32.75 μ l	3436.75 μ l
wax	90:10 Paraffin:Chillout™ 14.		

2. Pre-wax PCR tubes by melting the 90:10 Paraffin:Chillout™ 14 wax and adding the melted wax to the tubes in such a way that the wax solidifies on the sides of the upper half of the tubes.

15 3. Mix solutions by tapping and/or inverting the tubes (do not vortex). Add 40 μ l QPCR mix to the pre-waxed PCR tubes. Add the solution one tube at a time carefully avoiding the sides and wax in the tubes. Note that it is important to keep the QPCR and the Qlig mixes separate as any QPCR mix in the ligation and the reaction will not work.

20 4. The tubes are placed in a thermal cycler without lids and the wax is melted onto the liquid layer by incubating at 75°C for 2 min, followed by decreasing increments of 5°C for every 2 min until 25°C is reached.

25 5. Combine the following components for the Qlig mix as shown:

30

Reagent	Concentration	1 rxn	24 rxn
RE 1	depends on RE	0.2 μ l	5.2 μ l
RE 2	depends on RE	0.2 μ l	5.2 μ l
5 Adapter set 1	20 pmole/ml for primer	1 μ l	26 μ l
Adapter set 2	20 pmole/ml for primer	1 μ l	26 μ l
ATP	10 mM	0.8 μ l	20.8 μ l
10 NEB 2	10X	1 μ l	26 μ l
Betaine	5 M	2 μ l	52 μ l
Ligase	1 U/ml	0.2 μ l	5.2 μ l
H ₂ O		2.6 μ l	67.6 μ l

15 The amount for 24 rxns is advantageous for 8 cDNAs reactions done in triplicate.

20 6. After the Qlig mixes are complete for each set of enzymes the mix can be split up into tubes before adding the cDNAs. 24 reactions can be split up into 8 tubes each with 3 reaction volumes (approximately 27 μ l).

7. Add the cDNA to the tubes and mix:

Reagent	Concentration	1 rxn	3 rxns
25 cDNA sample	1 ng/ μ l	1 μ l	3 μ l

The cDNA is prediluted to the appropriate concentration of 1 ng/ μ l.

30 8. Add 10 μ l of the Qlig mix to the top of the wax being careful not to disturb the wax. In the case where 24 Qlig reactions are triplicated, the products can be split into 24 individual QPCR reactions.

9. Gently add the caps to the tubes. Excess pressure can 35 disturb the wax.

10. Place the tubes in a thermal cycler and perform the following thermal protocol.

Temp (in °C)	Time (in min.)	Reaction
37	30	Optimal RE digestion temperature
5 Ramp down to 37°C at -1°C/min.		
16	60	Optimal ligation temperature
37	15	Optimal RE digestion temperature
10 72	20	Melt wax; mix solutions in tube; blunt-end chains
Cycle the following steps for the number of PCR cycles, preferably 20		
96	30 sec.	Denaturing
15 57	1	Hybridizing
72	2	Chain elongation
End of the PCR cycles		
72	10	
20 4	hold	

20

11. After program is finished heat the tubes to 75°C for 5 minutes. Pull out the tubes and immediately turn them upside down till the wax hardens.
12. Place finished reactions in freezer or proceed directly to further processing.

25 The following are the preferred vendors for the various reagents used in this protocol.

30

35

R agents	Vendor	Catalog #
Enzymes	NEB (Beverly, MA)	
5 Adapters	Amitof/NBI (Allston, MA)	(see Table 10 for sequences)
Fluorescent Primers	Genosys (The Woodlands, TX)	(see Table 10 for sequences)
10 ATP	Pharmacia (Newark, NJ))	27-1006-02
dNTP	Pharmacia	27-2035-02
Klentag	Ab peptides (St. Louis, MO)	1001
15 PFU	Stratagene (Los Angeles, CA)	600154
Betaine	Sigma (St. Louis, MO)	B-2754
20 Paraffin wax	Fluka Chemical, Inc. (Ronkonkoma, N.Y.)	76243
Chillout™ 14 liquid wax	MJ Research	
Ligase	BRL (Baltimore, MD)	15224-025

6.4.2. POST AMPLIFICATION CLEANUP PROTOCOL AND OTHER STEPS

25 Different post-amplification steps are appropriate for the various embodiments of QEA™/RE embodiment. In one case, QEA™ reaction are performed with labeled primers having no conjugated capture moieties. In this case, QEA™ reaction products are simply separated by length. When separation is 30 by electrophoresis, the reaction products are suspended in a loading buffer and then loaded into an electrophoresis gel. A preferable electrophoresis apparatus is an ABI 377 (Applied Biosystems, Inc.) automated sequencer using the Gene Scan software (ABI) for analysis. The electrophoresis can be done 35 under non-denaturing conditions, in which the dsDNA remains together and carries the labels (if any) of both primers. It can also be done under denaturing conditions, in which each

ssDNA is separately labeled but typically are expected to migrate together.

In another case, one of the primers has a conjugated capture moiety, e.g., biotin, either for post-amplification cleanup prior to separation or as part of the SEQ-QEA™ embodiment. In this case, QEA™ reaction products are first subject to a cleanup protocol for removing excess reagents and certain reaction products.

The following buffers are used in the post-PCR cleanup protocol.

Binding Buffer (H₂O solution)

- I. 5 M NaCl
- II. 10 mM Tris, pH 8.0
- III. 1 mM EDTA

Wash Buffer (H₂O solution)

15

- I. 10 mM Tris, pH 8.0
- II. 10 mM EDTA

Loading Buffer (denaturing)

- I. 80% deionized formamide
- II. 20% 25 mM EDTA (pH 8.0), 50 mg/mL Blue dextran

20

- Ladder Loading Buffer
- I. 100 μL Gene Scan 500 ROX with 900 μL Loading Buffer

Post-PCR Cleanup Protocol:

25

1. Prepare enough streptavidin magnetic beads for purifying QEA™ products (Catalog No. MSTR0510 of CPG, Lincoln Park, N.J.). Use 3 μL of beads for every 5 μL of QEA™ reaction product. Pre-wash beads in final suspension volume with binding buffer.

30

35

	1 Reaction		96 Reactions		
	Sample Volume	Bead Volume	Suspension Volume	Bead Volume	Suspension Volume
5	5 μ l	3 μ l	10 μ l	300 μ l	1 ml
	10 μ l	6 μ l	10 μ l	600 μ l	1 ml
	15 μ l	9 μ l	10 μ l	900 μ l	1 ml
	20 μ l	12 μ l	10 μ l	1200 μ l	1 ml

10 2. Dispense 10 μ L of washed beads for every QEA™ sample to be processed. Purifications are done in a 96 well Falcon TC plate.

3. Add QEA™ product to beads. Mix well and incubate 30 minutes at 50°C.

15 4. Bring volume of sample up to 100 μ l with binding buffer. Place plate on 96 well magnetic particle concentrator. Allow beads to migrate for 5 minutes.

5. Remove liquid, add 200 μ L of washing buffer (TE pH 7.4).

6. Repeat the washing step 5.

20 7. In the case of a SEQ-QEA™ embodiment, the washed beads are now passed to the further steps of this embodiment as described in Sec. 6.5. In the other case of an embodiment using post-amplification cleanup alone, the washed beads are passed to the analysis step 9.

25 8. Optionally, the beads may be stored by passing to step 10.

8. For analysis the beads are resuspended in loading buffer (5 μ l for 5 μ l of beads). Gene Scan 500 ROX ladder can be mixed in a one-tenth dilution. The supernatant is then analyzed by electrophoresis under denaturing conditions.

30 9. In case the beads are to be stored, remove liquid and air dry the beads.

10. Store plate dry in at -20°C.

35

In the case where one of primers has a conjugated biotin moiety, QEA™ reaction products fall into the following three categories:

- 5 a) A dsDNA molecule of which neither strand has a biotin moiety;
- b) A dsDNA molecule having only one having with a conjugated biotin moiety;
- c) A dsDNA molecule having biotin moieties conjugated to both strands.

10 Category "a" products are not bound to the beads, and after the washing steps 5 and 6 of the previous protocol, they are washed from the beads, leaving only categories "b" and "c" attached to the beads. After step 9 in which the beads are resuspended in denaturing loading buffer, for category "b" 15 products, the strand not having the biotin moiety is released while the other strand with the biotin moiety is retained by the beads. For category "c" products, both strands are retained. Consequently, the electrophoresis of step 9 separates single strands deriving from those reaction 20 products having only one conjugated biotin moiety.

6.4.3. THE 5'-QEA™ EMBODIMENT

This subsection describes an exemplary protocol for QEA™ embodiments which generates cDNA fragments which on the 25 5' end are fixed with respect to the 5' cap of the source mRNA and which on the 3' end are singly cut by a chosen RE. First, input cDNA is synthesized according to the protocol of Sec. 6.3.3, or an equivalent protocol. Second, the protocols in Sec. 6.4.1 and 6.4.2 previously described, except 30 differing only in the composition of the Qlig mix, are performed.

1. cDNA is synthesized according to the protocol of Sec. 6.3.3.
- 35 2. The QPCR mix is prepared according to steps 1 through 4 of the protocol of Sec. 6.4.1.

3. Combine the following components for the Qlig mix as shown:

Regent	Concentration	1 rxn	24 rxn
RE 1	depends on RE	0.2 μ l	5.2 μ l
Adapter set 1	20 pmole/ml for primer	1 μ l	26 μ l
MA24 primer biotin-labeled	20 pmole/ml for primer	1 μ l	26 μ l
ATP	10 mM	0.8 μ l	20.8 μ l
NEB 2	10X	1 μ l	26 μ l
Betaine	5 M	2 μ l	52 μ l
Ligase	1 U/ml	0.2 μ l	5.2 μ l
H ₂ O		4.6 μ l	119.6 μ l

The amount for 24 reactions is advantageous for * reactions performed in triplicate.

4. The RE/ligase and PCR amplifications are processed according to steps 6 through 12 of the protocol of Sec. 6.4.1.

5. The reaction products are processed according to steps 1-6 and 8-10 of the cleanup protocol of Sec. 6.4.2.

25 After the washing step of the cleanup protocol, step 6, attached to the streptavidin beads are only products which are singly cut on the 3' end and are terminated at the 5' end by the biotin-labeled primer, which is ligated in a fixed relation to the 5' cap of the source mRNA. Thus, upon 30 denaturing electrophoresis, step 9 of the cleanup protocol, subsequent detection finds only signals from the desired singly cut end fragments of definite length.

35 6.4.4. FIRST ALTERNATIVE RE/LIGASE & AMPLIFICATION REACTIONS
The section describes less preferred protocols suitable for either manual or automated execution in two

tubes and suitable for labeled primers without a conjugated capture moiety. Otherwise the REs and the primer/linker components are chosen as previously described.

5 Adapter Annealing

Adapters are formed by annealing 12-mer linkers and 24-mer primers with some linker excess according to the following protocol:

- 10 1. Add to water linker and primer in a 2:1 concentration ratio (12-mer : 24-mer) with the primer at a total concentration of 5 pM per μ l.
2. Incubate at 50°C for 10 minutes.
3. Cool slowly to room temperature and store at -20°C.

15

Because there is no primer excess, primers must be separately added to the PCR reaction mix.

Manual RE/Ligase & Amplification Reactions

- 20 20 This protocol is advantageously applied to separate manually performed RE/Ligase and amplification reactions. First, the RE/ligase reaction is prepared for use in a 96 well thermal cycler. Add per reaction:

- 25 1. 1 U of chosen REs (New England Biolabs, Beverly, MA) (preferred RE pair listing in Sec. 6.10)
2. 1 μ l of pre-annealed adapters appropriate for the chosen REs are prepared as above
3. 1 μ l of Ligase/ATP (0.2 μ l T4 DNA ligase [1 U/ μ l]/0.8 μ l
- 30 30 10 mM ATP from Life Technologies (Gaithersburg, MD))
4. 0.5 μ l 50 mM MgCl₂
5. 10 ng of subject prepared cDNA
6. 1 μ l 10X NEB 2 buffer from New England Biolabs (Beverly, MA)
- 35 7. Water to bring total volume to 10 μ l

Then perform the RE/ligation reaction by following the thermal profile in Fig. 16A using a PTC-100 Thermal Cycler from MJ Research (Watertown, MA).

Next for the PCR amplification reaction mix by 5 combining:

1. 10 μ l 5X E-Mg (300 mM Tris-HCl pH 9.0, 75 mM $(\text{NH}_4)_2\text{SO}_4$, no Mg ions))
2. 100 pm of appropriate fluorescently labeled 24-mer 10 primers
3. 1 μ l 10 mM dNTP mix (Life Technologies, Gaithersburg, MD)
4. 2.5 U of 50:1 Taq polymerase (Life Technologies, Gaithersburg, MD) : Pfu polymerase (Stratagene, La 15 Jolla, CA)
5. Water to bring volume to 40 μ l per PCR reaction

Then perform the following steps:

- 20 1. Add 40 μ l of the PCR reaction mix to each RE/ligation reaction
2. Perform the PCR temperature profile of Fig. 16B using a PTC-100 thermal cycler (MJ Research, Watertown, MA)

25

Automated RE/Ligase & Amplification Reactions

The preceding protocol can be advantageously automated according to the current protocol which requires intermediate reagent additions. Reactions are preformed in a 30 standard 96 well thermal cycler format using a Beckman Biomek 2000 robot (Beckman, Sunnyvale, CA). Typically 4 cDNA samples are analyzed in duplicate with 12 different RE pairs, for a total of 96 reactions. All steps are performed by the robot, including solution mixing, from user provided stock 35 reagents, and temperature profile control.

Pre-annealed adapters are prepared as in the preceding section. Mix per RE/ligase reaction:

1. 1 U of appropriate RE (New England Biolabs, Beverly, MA)
2. 1 μ l of appropriate annealed adapter prepared as above (10 pm)
3. 0.1 μ l T4 DNA ligase [1 U/ μ l] (Life Technologies
- 5 (Gaithersburg, MD)
4. 1 μ l ATP (Life Technologies, Gaithersburg, MD)
5. 5 ng of subject prepared cDNA
6. 1.5 μ l 10X NEB 2 buffer from New England Biolabs (Beverly, MA)
- 10 7. 0.5 μ l of 50 mM MgCl₂,
8. Water to bring total volume to 10 μ l and transfer to thermal cycler

The robot requires 23 minutes total time to set up
15 the reactions. Then it performs the RE/ligation reaction by following the temperature profile of Fig. 16C using a PTC-100 Thermal Cycler equipped with a mechanized lid from MJ Research (Watertown, MA).

Next, prepare the PCR reaction mix by combining:

- 20 1. 10 μ l 5X E-Mg (300 mM Tris-HCl pH 9.0, 7.5 mM (NH₄)₂SC₁₀)
2. 100 pm of appropriate fluorescently labeled 24-mer primer
3. 1 μ l 10 mM dNTP mix (Life Technologies, Gaithersburg,
- 25 MD)
4. 2.5 U of 50:1 Taq polymerase (Life Technologies, Gaithersburg, MD) : Pfu polymerase (Stratagene, La Jolla, CA)
5. Water to bring volume to 35 μ l per PCR reaction

30 Preheat the PCR mix to 72°C and transfer 35 μ l of the PCR mix to each digestion/ligation reaction and mix. The robot requires 6 minutes for the transfer and mixing. Perform the RE/ligase and PCR amplification reactions
35 according to the temperature profile of Fig. 16B using a PTC-100 thermal cycler equipped with a mechanized lid (MJ Research, Watertown, MA).

The total elapsed time for the digestion/ligation and PCR amplification reactions is 179 minutes. No user intervention is required after initial experimental design and reagent positioning.

5

6.4.5. SECOND ALTERNATIVE RE/LIG. & AMPLIFICATION REACTIONS

The section describes a much less preferred fully manual protocol in which the RE, the ligation, and the PCR amplification reactions are all separately performed in three 10 tubes. It is suitable for labeled primers without a conjugated capture moiety, with the REs and the primer/linker components otherwise chosen as previously described. It is a less preferred protocol.

15 RE Digestion Reaction

1. Mix the following reagents
 - 0.5 μ l prepared cDNA (100 ng/ μ l) mixture (total 50 ng of cDNA)
 - 10 μ l New England Biolabs Buffer No. 2
 - 20 3 Units RE enzyme
2. Incubate for 2 hours at 37°C.

Larger size digests with higher concentrations of cDNA can be used and fractions of the digest saved for 25 additional sets of experiments.

Adapter Ligation

Since it is important to remove unwanted ligation products, such as concatamers of fragments from different 30 cDNAs resulting from hybridization of RE sticky ends, the restriction enzyme is left active during ligation. This leads to a continuing cutting of unwanted concatamers and end ligation of the desired end adapters. The majority of restriction enzymes are active at the 16°C ligation 35 temperature. Ligation profiles consisting of optimum ligation conditions interspersed with optimum digestion conditions can also be used to increase efficiency of this

process. An exemplary profile comprises periodically cycling between 37°C for 2 hours and 16°C for 2 hours at a ramp of 1°C/min.

One linker complementary to each 5 minutes overhang generated by each RE is required. 100 picomoles ("pm") is a sufficient molar excess for the protocol described. For each linker a complementary uniquely labeled primer is added for ligation to the cut ends of cDNAs. 100 pm is a sufficient molar excess for the protocol described. If the amounts of RE cDNA is changed the linker and primer amounts should be proportionately changed.

Ligation Reaction (per 10 µl and 50 ng cDNA)

1. Mix the following reagents

15	<u>Component</u>	<u>Volume</u>
	RE digested cDNA mixture	10 µl
	100 pN/µl each primer	1 µl
	100 pM/µl each linker	1 µl

20 2. Thermally cycle from 50°C (a temperature at which the primers and linkers hybridize) to 10°C (-1°C/minute) then back to 16°C

3. Add 2 µl 10 mM ATP with 0.2 µl T4 DNA ligase (Premix 0.1 µl ligase 1 U/µl per 1 µl ATP) (E. Coli ligase is a less preferred alternative ligase.)

25 4. Incubate 12 hours at 16°C. This step can be shortened to less than 2 hours with proportionately higher ligase concentration. Alternately the thermal cycling protocol described can be used here.

30 5. Incubate 2 hours 37°C

6. Incubate 20 minutes at 65°C to heat inactivate the ligase (last step should be RE cutting).

7. Hold at 4°C

35 Amplification Of Fragments With Ligated Adapters

This step amplifies the fragments that have been cut twice and ligated with adapters unique for each RE cut end. It is designed for a high amplification specificity. Multiple amplifications are performed, with an increasing 5 number of amplification cycles. Use of the minimum number of cycles to get the desired signal is preferred. Amplifications above 20 cycles are not generally reliably quantitative.

Mix the following to form the ligation mix:

10

	<u>Component</u>	<u>Volume</u>
	RE/Ligase cDNA mixture	5 μ l
	10X PCR Buffer	5 μ l
	25 mM MgCl ₂	3 μ l
15	10 mM dNTPs	1 μ l
	100 pM/ μ l each primer	1 μ l

Mix the following to form 150 μ l PCR-Premix

20

	<u>Volume</u>	<u>Component</u>
	30 μ l	Buffer E (ligation mix will contribute 0.3 mM MgCl ₂)
	1 μ l	(300 pm/ μ l Rbuni24 Flour) 24 mer primer strand (50 pm/ μ l NBuni24 Tamra)
25	0.6 μ l	Taq polymerase (per 150 μ l)
	3 μ l	dNTP (10 mM)
	106 μ l	H ₂ O

30 Amplification of fragments is more specific if the small linker dissociates from the ligated primer-cDNA complex prior to amplification. The following is an exemplary method for amplification of the results of six RE/ligase reactions.

35 1. Place three strips of six PCR tubes, marked 10, 15, and 20 cycles, into three rows on ice as shown.

20 cycles	1	2	3	4	5	6	-Add 140 μ l PCR-premix
15 cycles	1	2	3	4	5	6	
5 10 cycles	1	2	3	4	5	6	-Add 10 μ l ligation mix

2. Place 10 μ l ligation mix in each tube in 10 cycle row
 3. Place 140 μ l PCR premix in each tube in 20 cycle row
 10 4. Place into cycler and incubate for 5 minutes at 72°C. This melts linker which was not covalently ligated to the second strand of a cDNA fragment and allows the PCR premix to come to temperature.
 5. Move the 140 μ l PCR premix into the tubes in the 10 cycle
 15 row containing the 10 μ l ligation mix, then place 50 μ l of result into corresponding tubes each in other rows.
 6. Incubate for 5 minutes at 72°C. This finishes
 incompletely double stranded cDNA ends into complete
 dsDNA, the top primer being used as template for second
 20 strand completion.

The amplification cycle is designed to raise specificity and reproducibility of the reaction. High temperature and long melting times are used to reduce bias of 25 amplification due to high G+C content. Long extension times are used to reduce bias in favor of smaller fragments. Long denaturing times reduce PCR bias due to melting rates of fragments, and long extension time reduces PCR bias on fragment sizes.

30

1. Thermally cycle 95°C for 1 minute followed by 68°C for 3 minutes.
2. Incubate at 72°C for 10 minutes at end of reaction.

35 **6.4.6. OPTIONAL POST-AMPLIFICATION STEPS**

Several optional steps can improve the signal from the detected bands. First, single strands produced as a

result of linear amplification from singly cut fragments can be removed by the use of single strand specific exonuclease. Exo I is the preferred single strand specific nuclease, and is used by incubating 2 units of nuclease with the product of 5 each PCR reaction for 60 minutes at 37°C.

Second, the amplified products can be concentrated prior to detection either by ethanol precipitation or column separation with a hydroxyapatite column.

Several labeling methods are usable, including 10 fluorescent labeling as has been described, silver staining, radiolabelled end primers, and intercalating dyes. Fluorescent end labeling is preferred for high throughput analysis with silver staining preferred if the individual bands are to be removed from the gel for further processing, 15 such as sequencing.

Finally, fourth, use of two primers allows direct sequencing of separated strands by standard techniques. Also separated strands can be directly cloned into vectors for use in RNA assays such as in situ analysis. In that case, it is 20 more preferred to use primers containing T7 or other polymerase signals.

25

30

35

6.5. PREFERRED SEQ-QEA™ METHOD

The SEQ-QEA™ embodiment is practiced with the special SEQ-QEA™ primers. One SEQ-QEA™ primer in a reaction has a Type IIS restriction enzyme (e.g., FokI) recognition site and a fluorescent tag, (e.g., FAM (carboxy-fluorescein) (ABI)) attached at the 5' end. The other primer used has a biotin capture moiety ("Bio") and comprises either a uracil residue or a site for a rare-cutting restriction enzyme like AscI. Sec. 6.10.1 and Table 18 has a list of exemplary 10 primers and linkers for the SEQ-QEA™ methods.

Using these primers with corresponding linkers and appropriate REs, the preferred QEA™ protocol of Sec. 6.4.1 is performed and is followed by the post-PCR cleanup protocol of Sec. 6.4.2 through the step 6 washing. As noted in step 7, 15 the products of step 6 are input to the further steps of the SEQ-QEA™ embodiment.

The following are preferable primers and linkers to be used together with the an RE1 of BglII and an RE2 of EspHI.

20	<u>SEQ-QEA™ method primer pairs</u>	<u>Type-IIS Enzyme</u>	<u>Method of Bead Release</u>
1)	KA5/KA24-FAM + RC9/UC24-Bio	FokI	UDG
2)	BA5/BA24-FAM + RC9/UC24-Bio	BbvI	UDG
3)	KA5/KA24-FAM + RC9/SC24-Bio	FokI	AscI
25	4) BA5/BA24-FAM + RC9/SC24-Bio	BbvI	AscI

Using the above REs and primer pairs, QEA™ method reaction products obtained fall into the following three categories:

- a) A double-stranded DNA with a 5' FAM label with nearby 30 sequence containing a recognition site for FokI or BbvI on one strand, and a 3' biotin label with nearby sequence containing a uracil residue or an AscI recognition site on the other strand (in the case where different REs cut at each end)
- 35 b) A double-stranded DNA with a 5' biotin label with nearby sequence containing a uracil residue or an AscI recognition site on one strand, and a 3' biotin label

with nearby sequence containing a uracil residue or an AscI recognition site on the other strand (in the case where same RE cuts at both ends)

5 c) A double-stranded DNA with a 5' FAM label with nearby sequence containing a recognition site for FokI or BbvI on one strand, and a 3' FAM label with nearby sequence containing a recognition site for FokI or BbvI on the other strand (in the case where same RE cuts at both ends)

10 Typically, after QEA™ reactions according to the protocol of Sec. 6.4.1 is completed, 45 μ l out of 50 μ l is processed (the rest is saved). During the post-PCR cleanup according to the protocol of Sec. 6.4.2, these 45 μ l of the reaction products are bound to the magnetic streptavidin 15 beads and washed at step 6 of this protocol. After this step, only category "a" and "b" products are retained by the magnetic streptavidin beads, the category "c" products having no biotin moieties. Subsequently, the DNA bound to the beads is digested with the Type IIS restriction enzyme in a volume 20 of 100 μ l of a suitable 1X RE buffer, e.g. NEB 4 for FokI, with about 10 units of the enzyme for 3 hours at 37°C. After Type IIS RE digestion and washing only category "a" products are retained by the beads, the category "b" products having been cut at both ends and released from the beads. The 25 supernatant is then removed and the beads are washed three times with the wash buffer. Type IIS restriction enzymes cleave DNA at a location outside their recognition sites, thus producing overhangs of unknown sequences (Szybalski et al., 1991, *Gene* 100:13-26). The Type IIS digestion thus 30 releases the FAM label of the category "a" products and creates a fragment-specific overhang that acts as a template for sequencing. Complete Type IIS digestion can be checked for by the absence of the FAM label.

The end-sequencing reaction is essentially a chain 35 fill-in reaction using the overhang generated by the Type-IIS restriction enzyme as a template. Dideoxy chain terminators labeled with different ABI fluorescent dyes are mixed at high

ratios with dNTPs to ensure high frequency of incorporation, and the DNA polymerase enzyme used (e.g., Sequenase (T7 DNA polymerase), Taquenase (Taq polymerase)) has high affinity for the labeled dideoxynucleotides. A sequencing mix 5 totalling 20 μ l containing the appropriate 1X buffer, 1 μ l dNTPs diluted 1/200 from stock (3 mM dATP, 1.2 mM dCTP, 4.5 mM dGTP, 1.2 mM dTTP), 0.5 μ l each ABI dye-labeled terminator solution (containing ddATP, ddCTP, ddGTP and ddTTP, respectively), (and 1 μ l 0.1 M DTT for Sequenase) is 10 made. The beads are resuspended in the sequencing mix and 0.1 μ l Taquenase is added and the reaction is incubated at 65°C for 15 minutes. If Sequenase is to be used, 0.1 μ l Sequenase is added instead of Taquenase and the reaction is 15 incubated at 37°C for 15 minutes. After this, the reaction mix is transferred to a magnet and the supernatant is removed. The beads are washed twice with wash buffer I.

The above-described end-sequencing reaction incorporates dye labeled nucleotides into the strand that contains biotin. Since biotin-streptavidin binding is nearly 20 irreversible, the labeled strands must be cleaved for analysis by electrophoresis. This is achieved by treating UMP-containing fragments with Uracil DNA Glycosylase (UDG), or cleaving AsCl-site-containing fragments with AsCl. UDG removes the Uracil residue from dsDNA; the phosphate backbone 25 is subsequently hydrolyzed at temperatures above room temperature and at pH>8.3.

For UDG treatment, the beads are resuspended in 20 μ l UDG buffer (30 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂), 0.2 units of UDG are added and the reaction is incubated at 30 room temperature for 30 minutes. The reaction is then transferred to a magnet and the supernatant removed. The biotinylated strand, which is the strand that is being filled in during end-sequencing, is still attached to the beads as UDG does not destroy the backbone, but makes it very 35 susceptible to hydrolysis.

The beads are resuspended in 5 μ l formamide loading buffer. These are then split into 2 tubes of 2.5 μ l each.

Another 2.5 μ l formamide loading buffer is added to one and 2.5 μ l formamide loading buffer with 20% GS500 ROX ladder (ABI) is added to the other. These are heated at 95°C for 5 minutes to effect hydrolysis and denaturation. Then they are 5 electrophoretically separated.

In case of the biotinylated primer having an AsCI site, the following is performed. The beads are resuspended in 20 μ l of AsCI buffer (15 mM KOAc, 20 mM Tris, 10 mM MgOAc, 1 mM DTT, pH 7.9) and 5 units of AsCI is added and incubated 10 at 37°C for 1 hour. The beads are separated on a magnet and the supernatant that contains the digestion products is precipitated with three volumes of ethanol after the addition of 5 μ g of glycogen. The pellet is resuspended in 5 μ l formamide loading buffer and split into 2 tubes of 2.5 μ l 15 each. Another 2.5 μ l formamide loading buffer is added to one and 2.5 μ l formamide loading buffer with 20% GS500 ROX ladder is added to the other. These are heated at 95°C for 5 minutes and analyzed by electrophoretic separation.

Sequencing is completed by gel electrophoretic 20 separation of released and sequenced strands. The overhang sequence is given by the order of partially filled in fragments observed.

6.6. QEA™ BY THE PCR EMBODIMENT

25 This is an alternative QEA™ embodiment based on PCR amplification of fragments between target subsequences recognized by PCR primers or sets of PCR primers. It is designed for the preferred primers described with reference to Fig. 5. If other primers are used, such as simple sets of 30 degenerate oligonucleotides, step 5, the first low stringency PCR cycle, is omitted.

First strand cDNA synthesis is carried out according to Sec. 6.3. PCR amplification with defined sets of primers is performed according to the following protocol.

1. RNase treat the 1st strand mix with 1 μ l of RNase Cocktail from Ambion, Inc. (Austin, TX) at 37°C for 30 minutes.
2. Phenol/CHCl₃ extract the mixture 2 times, and purify it on a Centricon 100, Millipore Corporation (Bedford, MA) using water as the filtrate.
- 5 3. Bring the end volume of the cDNA to 50 μ l (starting with 10 ng RNA/ μ l).
4. Set up the following PCR Reaction:

10

<u>Component</u>	<u>Volume</u>
cDNA (~10 ng/ μ l)	1 μ l
1.0X PCR Buffer	2.5 μ l
15 25 mM MgCl ₂	1.5 μ l
10 mM dNTPs	0.5 μ l
20 20 μ M/ μ l primer 1	2.5 μ l
20 20 μ M/ μ l primer 2	2.5 μ l
Taq Poly. (5 U/ μ l)	0.2 μ l
20 water	14.3 μ l

20

5. One low stringency cycle with the profile:
40°C for 3 minutes (annealing)
72°C for 1 minute (extension)
- 25 6. Cycle using the following profile:
95°C for 1 minute
15-30 times:
95°C for 30 seconds
50°C for 1 minute
72°C for 1 minute
- 30 35 72°C for 5 minutes
7. 4°C hold.
8. Samples are precipitated, resuspended in denaturing loading buffer, and analyzed by electrophoresis (either under denaturing or non-denaturing conditions).

35

6.7. EXAMPLE OF SIMULATED ANNEALING

From the October 1994 GenBank database containing human coding sequences, 12,000 of the first continuous coding domain sequences ("CDS") were selected as in Sec. 6.1. This 5 selection resulted in a selected database of sequences biased towards short sequences. Frequency tables were then created that listed the occurrence frequency of each nucleotide subsequence of lengths 4, 5, 6, 7, and 8 in this selected database. Test target subsequences were initially selected 10 whose probability of occurrence was near to 50%. This was feasible for the 4-mers, as they bind relatively frequently, but as the occurrence probability decreases with length, for longer sequences, the occurrence probability was often substantially less than 50%. These initially selected target 15 subsequences were then optimized, using the simulated annealing CC experimental design methods, to pick the best 16 subsequences.

Tables 5, 6 and 7 present the results for target subsequences of lengths 4, 5 and 6, respectively. Table 8 20 presents the results for optimizing target subsequences of length 4 through 6 together. Simulated annealing generally produced an approximately 20% improvement over a target subsequence selection guided only by the occurrence and independence probability criteria. This level of 25 optimization is likely to improve with larger and less redundant databases that represent longer genes. Longer sequences bind too infrequently in this database to make useful hash codes.

30

TABLE 5: AN OPTIMIZED SET OF 4-MER SUBSEQUENCES

35

CGTC	GTAA	ACTA	CTAG
TTTT	TGTA	AATC	GTTG
TACC	TTGT	TTCG	GATA
CGGT	CTCG	AACG	GGTA

The target subsequences in Table 5 were chosen from all possible 256 4-mers. There are 2.41 CDSs per hash code on average. There was 692 CDSs (out of 12000) which are not complementary to any of these subsequences.

5

TABLE 6: AN OPTIMIZED SET OF 5-MER SUBSEQUENCES

AGGCA	ACTGT	GTCTC	TGTGC
CAACT	GCCCC	ACTAC	GTGAC
GCACC	GTCTG	GCCTC	CAGGT
AGGGG	GGAAC	GCTCC	GCTCT

The target subsequences in Table 6 were chosen from the 300 most frequently occurring 5-mers. There are 2.33 CDSs per hash code on average. There was 829 CDSs (out of 12000) which are not complementary to any of these subsequences

20

TABLE 7: AN OPTIMIZED SET OF 6-MER SUBSEQUENCES

TCCTCA	CCAGGC	AGCAGC	CTCCTG
AGCTGG	CTCTGG	CCAGGG	CAGAGA
GCCTGG	ACTGGA	CACCAT	GCTGTG
ACTGTG	TCTGTG	CCAAGG	CCTGGA

25

The target subsequences in Table 7 were chosen from the 200 most frequently occurring 6-mers. There was 2.63 CDSs per hash code on average. There are 1530 CDSs (out of 12000) which are not complementary to any of these subsequences.

30

35

TABLE 8: AN OPTIMIZED SET OF 4-, 5-, AND 6-MER SUBSEQUENCES

CTCG	TTCG	GATA	TTTT
CTAG	GGTA	ACTGT	ACTAC
CAACT	GTCTG	AGGCA	GCACC
TGTGC	GGAAC	AGGGG	CTCCTG

The target subsequences in Table 8 were chosen from sets in Tables 1-3. There was 2.22 CDSs per hash code on average. There are 715 CDSs (out of 12000) which are not complementary to any of these subsequences.

The bias of the selected CDSs toward short sequences, on the average less than the length of a typical gene, partially explains the 5-10% of CDSs that were not complementary to any selected target subsequence. Longer sequences would be expected to have more hits as they have more variability. Also more target subsequences can be chosen to improve coverage. The 2.2 to 2.6 CDSs per individual hash code is partially explained by replication in the selected database. No attempt was made to insure each CDS is unique in the selected database.

6.8. QEA™ RESULTS

This subsection present results from QEA™ experiments directed primarily to the query and tissue modes.

6.8.1. QUERY MODE QEA™ RESULTS

The pattern of gene expression differs from tissue to tissue, and is modulated both during normal development and during the progression of many diseases, including cancer. Query mode QEA™ experiments were used to investigate differences in gene expression between normal, hyperplastic, and adenocarcinomatous glandular tissues. We had at our disposal voxels containing all three types of tissue, preserved in such a way that the adjacent tissue sections were available for later *in situ* hybridization. The following experiments were carried out with normal,

hyperplastic, and adenocarcinomatous tissue, respectively, of a particular gland.

RNA Extraction and cDNA Synthesis

5 Isolation of total RNA and poly(A)⁺ RNA from homogenized glandular tissue voxels was performed substantially as described in Sec. 6.3.1. cDNA was prepared substantially as described in Sec. 6.3.4.

10 Quantitative Expression Analysis

QEA™ reactions were performed by the preferred RE embodiment substantially as described in Sec. 6.4.4. This included the following steps.

15 Adapter Annealing

Pairs of 12-base and 24-base primers were pre-annealed at a ratio of 2:1 (12 mer : 24 mer) at a concentration of 5 picomoles 24 mer per microliter in 1X NEB 2 buffer. For linker/primer hybridization, the 20 oligonucleotide mixture was heated to 50°C for 10 minutes, and allowed to cool slowly to room temperature. For this experiment, 10 picomoles of JC3 and 5 picomoles of JC24, and 10 picomoles of RC6 and 5 picomoles of RC24 were separately pre-annealed. The sequences of JC3, JC24, RC6, and RC24 are 25 listed in Table 10 of Sec. 6.10, infra.

Restriction-Digestion/Ligation Reaction

Reactions were prepared in for use in a 8-well thermal cycler format. Glandular cDNA isolated from 10 30 separate voxels of tissue was cut with HindIII and NgoMI, and pre-annealed linkers were ligated onto the 4 base 5' overhangs that these enzymes generated. Added per each QEA™ reaction were:

35 1 Unit of HindIII (New England Biolabs, Beverly MA)
1 Unit of NgoMI (New England Biolabs, Beverly MA)
1 μl of pre-annealed JC3/JC24

1 μ l of pre-annealed RC6/RC24
1 μ l Ligase/ATP (0.2 μ l T4 DNA Ligase (1 Unit/ μ l) and
0.8 μ l 10 mM ATP - Life Technologies, Gaithersburg
MD)

5 0.5 μ l 50 mM MgCl₂
10 ng of glandular cDNA
1 μ l 10X NEB 2 Buffer (New England Biolabs, Beverly MA)
Total volume of 10 μ l with H₂O

10 The temperature profile of Fig. 16A was performed
using a PTC-100 Thermal Cycler (MJ Research, Watertown MA).

Amplification Reaction

The products of the RE/ligation reaction were then
15 amplified using RC24 and JC24 primers. The PCR reaction mix
included:

10 μ l 5X E-Mg (300 mM Tris-HCl pH 9.0, 75 mM (NH₄)₂SO₄)
100 pm RC24
20 100 pm JC24
1 μ l 10 mM dNTP mix (Life Technologies, Gaithersburg MD)
2.5 Units 50:1 Taq polymerase (Life Technologies,
Gaithersburg MD): Pfu polymerase (Stratagene, La
Jolla CA) mix
25 Total volume of 40 μ l with H₂O.

40 μ l preheated PCR reaction mix was added to each
restriction-digestion/ligation reaction. The temperature
profile of Fig. 16B was performed using a PTC-100 Thermal
30 Cycler (MJ Research, Watertown MA).

QEA™ Analysis

The reaction products were separated on a 5%
acrylamide sequencing gel, and detected by silver staining.
35 Lane-to-lane comparisons were made both by visual inspection
of the gel, and by comparing computer enhanced images
obtained from scanning the gel using standard computer

scanner equipment. One particular band of length X bp was differentially expressed, being prominent in some samples but absent in others. This band was picked from the gel, PCR re-amplified, and sequenced.

5 QEA™ analysis was performed substantially as described in Sec. 5.4.1 using the CDS database constructed as described in Sec. 6.1. Four possible sequences in that database were found to be possible contributors to a fragment of Y bp (note that Y bp = X - 46 bp, where PCR primers add 46
10 bp to the fragment length), sequences A, B, C, and D. Analysis of the sequencing of the picked band confirmed that this DNA fragment was produced by sequence C, which is presently entered in GenBank. This result confirms the correct functioning of the integrated experimental and
15 analysis methods.

Further, analysis of sequence C predicted that a second double-digest, using REs BspHI and BstYI, would yield a second, non-overlapping restriction fragment at Z bp in length (plus the 46 bp of ligated primers). A second QEA™
20 reaction was performed using these glandular cDNAs. The previously described experimental conditions were used, with the exception of substituting BspHI, BstYI, RA5/RA24 and JC9/JC24 for HindIII, NgoMI, JC3/JC24 and RC6/RC24 during the RE/ligation reaction and of substituting RA24 and JC24 during
25 amplification reaction. Analysis of the results of this second QEA™ experiment on silver-stained acrylamide gels, as above, revealed the presence of a band of the predicted size, Z + 46 bp, that was also differentially expressed in the same tissue samples as the X bp fragment. This results confirms
30 the correct functioning of the mock digest prediction methods coupled with subsequence actual experimental digest.

Additional hybrid primers were designed to facilitate direct sequencing of QEA™ products and the direct generation of RNA probes for the *in situ* hybridization to the
35 original tissue sample. The M13-21 primer or the M13 reverse primer (in italics) were fused to the first 23 nucleotides of

JC24 and RC24 (in bold), respectively, to allow direct sequencing of the double-digested QEA™ products.

M13-21J + JA24: 5' **GGC GCG CCT GTA AAA CGA CGG CCA GTA**
5 CCG ACG TCG ACT ATC CAT GAA G 3' (SEQ ID NO:56)
M13revR + RA24: 5' **AAA ACT GCA GGA AAC AGC TAT GAC CAG**
CAC TCT CCA GCC TCT CAC CGA 3' (SEQ ID NO:57)

In order to enable direct generation of anti-sense RNA probes 10 for *in situ* hybridization, the phage T7 promotor (in italics) was fused to the first 23 nucleotides of JA24/JC24 and RA24/RC24 (in bold).

T7 + JA24: 5' **ACT TCG AAA TTA ATA CGA CTC ACT ATA GGG ACC**
15 GAC GTC GAC TAT CCA TGA AG 3' (SEQ ID NO:58)
T7 + RA24: 5' **ACT TCG AAA TTA ATA CGA CTC ACT ATA GGG AGC**
ACT CTC CAG CCT CTC ACC GA 3' (SEQ ID NO:59)

6.8.2. TISSUE MODE QEA™ RESULTS

20 Isolation of Human Placental Lactogen using QEA™

Lactogen is one of the most highly expressed genes in the human placenta and has a known sequence. The sequence of lactogen was retrieved from GenBank and mock digestion reactions were performed, substantially as described in § 25 5.4.1, with a wide selection of possible RE pairs. These mock digestions showed that digesting placental cDNA with the restriction enzymes BssHII and XbaI yields a lactogen fragment of 166 bp in length.

30 RNA Extraction and cDNA Synthesis

Isolation of total RNA and poly(A)+ RNA from homogenized human placenta tissue was performed substantially as described in Sec. 6.3.1. cDNA was prepared substantially as described in Sec. 6.3.4.

Quantitative Expression Analysis

QEA™ reactions were performed by the preferred RE embodiment substantially as described in Sec. 6.4.3. This included the following steps.

5

Adapter Annealing

Pairs of 12-base and 24-base primers were pre-annealed at a ratio of 2:1 (12 mer : 24 mer) at a concentration of 5 picomoles 24 mer per microliter in 1X NEB 10 2 buffer. The oligonucleotide mixture was heated to 50°C for 10 minutes, and allowed to cool slowly to room temperature. For this experiment, 10 picomoles of RC8 and 5 picomoles of RC24, and 10 picomoles of JC7 and 5 picomoles of JC24 were separately pre-annealed. The sequences of RC8, RC24, JC7, 15 and JC24 are set forth in Table 10 of Sec. 6.10, infra.

Restriction-Digestion/Ligation Reaction

Reactions were prepared for use in a 9-well thermal cycler format. Placental cDNA was cut with BssHII and XbaI, 20 and pre-annealed adapters ligated onto the 4 base 5' overhangs that these enzymes generated. Added per reaction were:

1 Unit of BssHII (New England Biolabs, Beverly MA)
25 1 Unit of XbaI (New England Biolabs, Beverly MA)
1 µl of pre-annealed RC8/RC24
1 µl of pre-annealed JC7/JC24
1 µl Ligase/ATP (0.2µl T4 DNA Ligase (1 Unit/µl) and
0.8µl 10 mM ATP - Life Technologies, Gaithersburg
30 MD)
0.5µl 50 mM MgCl₂
10 ng of placental cDNA
1µl 10X NEB 2 Buffer (New England Biolabs, Beverly MA)
Total volume of 10µl with H₂O.

35

The temperature profile of Fig. 16A was performed using a PTC-100 Thermal Cycler (MJ Research, Watertown MA).

Amplification Reaction

The products of the RE/ligation reaction were then amplified using RC24 and JC24 primers (see Table 10, infra). The PCR reaction mix included:

5

10 μ l 5X E-Mg (300 mM Tris-HCl pH 9.0, 75 mM (NH₄)₂SO₄)

100 pm RC24

100 pm JC24

1 μ l 10 mM dNTP mix (Life Technologies, Gaithersburg MD)

10 2.5 Units 50:1 Taq polymerase (Life Technologies, Gaithersburg MD): Pfu polymerase (Stratagene, La Jolla CA) mix.

Total volume of 40 μ l with H₂O.

15 40 μ l preheated PCR reaction mix was added to each restriction-digestion/ligation reaction. The temperature profile of Fig. 16B was performed using a PTC-100 Thermal Cycler (MJ Research, Watertown MA).

20 QEA™ Analysis

The reaction products were separated on a 5% acrylamide sequencing gel and detected by silver staining. A prominent band of size 212 bp was seen. This was predicted to correspond to the 166 bp lactogen BssHII-XbaI fragment, 25 with JC24 ligated to the BssHII site, and RC24 ligated to the XbaI site. To prove that this band did indeed correspond to lactogen, the 212 bp band was excised from the gel, re-amplified using JC24 and RC24, and the fragment was sequenced. Analysis of these sequencing results proved that 30 the fragment was from lactogen. Moreover, the lactogen sequence ended at the expected 4 base remnant of the restriction site, immediately followed by either JC24 (at the BssHII end) or RC24 (at the XbaI end).

This result confirmed the experimental design 35 methods of Sec. 5.4.2 applied to selection of a QEA™ experiment to identify certain sequences of interest, in this case the human placental lactogen sequence, in a tissue cDNA

sample. These design methods resulted in the selection of an experiment which successfully identified the gene intended.

Further QEA™ experiments were done according to the protocols of this section on human placental derived cDNA with differing enzyme combinations. One unit of each enzyme of the enzyme combinations listed in the first column of Table 9 were used in the restriction-digestion/ligation reaction protocol. Primers and linkers for each RE were chosen according to Table 10, with one appropriate "J" series linker and primer and one appropriate "R" series linker and primer used in each reaction. The reaction products were separated by electrophoresis on a 5% acrylamide gel and the bands detected by silver staining. Fragments from bands with the lengths listed in the second column of Table 9 were removed from the gel and sequenced. Sequencing identified the subsequences on the ends of the fragments and the precise lengths of each fragment. Each subsequence was characteristic of one of the REs used, confirming correct action of the ligation and amplification protocols. The third column of Table 9 lists end subsequences, with a "1" indicating the recognition subsequence of RE "Enz1" and a "2" indicating the recognition subsequence of RE "Enz2". Multiple fragments with the same length but differing recognition subsequence are placed in separate sub-rows in Table 9.

Mock digest reactions, as described in Sec. 5.4.1, were performed using the CDS database selected according to § 6.1. These mock digestion reactions searched this CDS database for sequences having recognition subsequences for the REs and such that the recognition subsequences are spaced apart in order to produce the fragments with the lengths listed. This search identified the database accession numbers listed in the fourth column of Table 9. The gene responsible for each accession number was determined from a GenBank lookup and is listed in the fifth column of Table 9. Table 9. Each such gene and its accompanying accession numbers is listed in a further sub-row. Multiple accession

numbers associated with one gene reflect the redundancy present in current CDS DNA sequence databases.

For all but one of the fragments recovered from the gel, the sequence for the fragment corresponded to one of the 5 genes identified by the mock digestion reaction as causing that fragment. This particular gene is indicated by displaying the gene name in underscore and bold in the fifth column of Table 9. That the gene determined by sequencing the separated fragment matched the prediction of the database 10 search confirms the efficacy of the experimental protocols and the computer implemented experimental analysis and ambiguity resolution methods of Sec. 5.4.1 and Sec. 5.4.3 for tissue mode QEA™. In fact, the mock digestion reactions provide a simple way of identifying possible ambiguities in 15 DNA sequence databases.

TABLE 9: PLACENTA GENE CALLS

	RE Combinations (Enz1 & Enz2)	Fragment Length	End Sub-seq.	Database Acc. Numbers	Gene Causing Fragment
20	BglII & BspE1	97	1,1	X07767	cAMP-Dependent Protein Kinase
			1,2	J03278, M21616	PDGF Receptor
				D23660, L20868, X73974	<u>Ribosomal Protein L4</u>
			2,2	M74096	Long Chain Acyl-CoA Dehydrogenase
25	BamH1 & BspE1	112	1,2	L26914, M93718, M95296	Nitric Oxide Synthase
				L22453, M90054, X73460	<u>Ribosomal Protein L3a</u>
30					
35	BglII & BspE1	115	1,2	M20496, X05256	<u>Cathepsin L</u>

	RE Combinations (Enz1 & Enz2)	Fragment Length	End Sub-seq.	Database Acc. Numbers	Gene Causing Fragment
5	BglIII & NgoM1	137	2,2	X55740	5'-Nucleotidase
		137	1,2	L18967	TRP2 Dopachrome Tautomerase
				L10386	Tranglutaminase E3
				S69231	Tyrosinease-Related Protein 2
				X56998, X56999	<u>Ubiquitin</u>
10	EcoRI & Bcl1	139	1,2	U14967	<u>Ribosomal Protein L21</u>
		144	1,2	J02984	<u>Ribosomal Protein S15</u>
				U04683, X80391	Olfactory Receptor OR17-40
15	Bcl1 & NgoM1	144	2,2	L12700	Engrailed-2
		144	1,2	X97234	Ribosomal Protein L11
				X14362	C3B/C4B Receptor
		146	1,2	M13932	<u>Ribosomal Protein S17</u>
20	BamHI & BspE1	166	1,2	J00118, V00573	<u>Lactogen</u>
		168	1,2	S56985, X63527	<u>Ribosomal Protein L19</u>
25	BssHII & XbaI	173	1,1	S59493, U10323	Nuclear Factor NF45
			1,2	M20882, M23575, M31125, M33666, M34420, M37399, M69245, M93061	<u>Pregnancy Sp. Glycoprotein beta 1</u>
30	BamHI & BspE1				
35					

RE Combinations (Enz1 & Enz2)	Fragment Length	End Sub-seq.	Database Acc. Numbers	Gene Causing Fragment
5 BglII & NgoM1	192	1,1	D26350	Inositol Triphosphatase Receptor
			L27711, L25876	Protein Phosphatase CIP2/KAP1
		1,2	D29992, L27624	<u>Tissue Factor Pathway Inhibitor 2</u>
BglII & AgeI	215	1,2	M11353, M11354	<u>Histone H3.3</u>

15 6.9. COLONY CALLING

The colony calling embodiment comprises the principal steps of cDNA library filter construction, PNA hybridization, and detection of hybridization. Determination of the sequence in a sample is done by the previously described computer implemented CC experimental analysis methods.

20 cDNA library filter construction

This protocol comprises three steps: first, robotic picking of colonies into microtiter plates, second, PCR amplification of inserts, and third, spotting of amplified cDNA inserts onto filters.

25 1. Colony picking -

30 a) Libraries are plated out at a density of 1,000-10,000 colonies per 100 mm Petri dish and are picked using a robot into 384 well microtiter plates containing 50 μ l of TB medium with the appropriate antibiotic. There are several commercially available robots to do this task. The preferable robot is from the Washington University Human Genome Sequencing Center (St. Louis, MO).

b) The picked colonies are grown for 8 hours at 37°C, and are frozen for archiving.

2. PCR amplification -

PCR primer pairs designed for insert amplification are dispensed with a standard 25 μ l PCR mix into 96 well microtiter plates. A 96 prong transfer tool picks and transfers samples to provide amplification templates from the 384 well colony into the 96 well PCR mixes. A standard 25 cycle amplification protocol generates 100-500 ng of insert DNA.

3. Spotting on filters -

The PCR products are pooled back into a 384 well format microtiter plates identical to the colony plates above. Spotting onto filters is a service performed by Research Genetics (Huntsville, AL).

Alternatively, cDNA library filters may be obtained from commercial sources in certain cases.

20 PNA hybridization and detection

PNAs are commercially available from Perseptive Biosystems (Bedford, MA). The protocol below uses 8 dyes on 16 different degenerate sets of PNA 8-mers containing as common subsequences the optimized 6-mer subsequences from Table 7. Thereby, complete classification and determination of expressed genes in a human tissue can be done with only 4 hybridizations generating a code of length 32. Actual conditions for stringency may vary depending on the PNA set used.

30

1. Hybridization -

A pool of 8 PNAs are used, labeled with 8 different fluorochromes made up at a concentration of 0.1 μ g/ml in 10 mM Phosphate buffer, pH 7.0, 1X Denhardt's solution (20 mg/ml Ficoll 400, polyvinylpyrrolidone, and BSA). The arrayed filters are hybridized for 16 hrs at 25° C, and

washed 3 times in the above buffer without PNAs at a temperature which maximizes signal/noise.

2. Visualization -

A fluorescent detection system, such as used for DNA analysis, can be used to distinguish the dyes, and thus the PNAs, present at each filter hybridization position. PNA presence or absence defines a code for each hybridization position on the filter.

10

6.10. PREFERRED QEA™ ADAPTERS AND REs PAIRS

Table 10 lists preferred primer-linker pairs that may be used as adapters for the preferred RE embodiment of QEA™. The primers listed cover possible double-digest RE combinations involving the approximately 56 available REs generating a 5' 4 bp overhang. There are 40 such REs available from New England Biolabs. For each QEA™ double digest reaction, one primer and one linker from the "R" series corresponding to one of the pair of REs and one primer and one linker from the "J" series corresponding to the other of the pair of REs are used together. This choice satisfies the adapter characteristics previously described. Two pairs from the same series are not compatible during amplification.

TABLE 10: SAMPLE ADAPTERS

25	Series	Adapter: Primer (longer strand) Linker (shorter strand)	RE
30		Notes: 'm' signifies an optional label or capture moiety.	
RA24		5' m-AGC ACT CTC CAG CCT CTC ACC GAA 3' (SEQ ID NO:1)	
RA1		3' AG TGG CTT TTAA	Tsp509 I MfeI EcoRI
RA5		3' AG TGG CTT GTAC	NcoI BspHI
RA6		3' AG TGG CTT GGCC	XmaI NgoMI BspEI

	RA7 (SEQ ID NO:5)	3' AG TGG CTT GCGC	BssHII Ascl
5	RA8 (SEQ ID NO:6)	3' AG TGG CTT GATC	AvrII NheI XbaI
RA9 (SEQ ID NO:7)	3' AG TGG CTT CTAG	DpnII BamHI BclI	
10	RA10 (SEQ ID NO:8)	3' AG TGG CTT CGCG	KasI
RA11 (SEQ ID NO:9)	3' AG TGG CTT CCGG	EagI Bsp120I NotI EaeI	
15	RA12 (SEQ ID NO:10)	3' AG TGG CTT CATG	BsiWI Acc65I BsrGI
RA14 (SEQ ID NO:11)	3' AG TGG CTT AGCT	XbaI SalI	
RA15 (SEQ ID NO:12)	3' AG TGG CTT ACGT	ApaLI	
20	RA16 (SEQ ID NO:13)	3' AG TGG CTT AATT	AflII
RA17 (SEQ ID NO:14)	3' AG TGG CTT AGCA	BssSI	
25	RC24 (SEQ ID NO:15)	5' m-AGC ACT CTC CAG CCT CTC ACC GAC 3'	
RC1 (SEQ ID NO:16)	3' AG TCG CTG TTAA	Tsp509I EcoRI ApoI	
30	RC3 (SEQ ID NO:17)	3' AG TCG CTG TCGA	HindIII
RC5 (SEQ ID NO:18)	3' AG TCG CTG GTAC	BspHI	
35	RC6 (SEQ ID NO:19)	3' AG TCG CTG GGCC	AgeI NgoMI BspEI SgrAI BsrFI BsaWI

	RC7 (SEQ ID NO:20)	3' AG TCG CTG GCGC	MluI BssHII AsCI
5	RC8 (SEQ ID NO:21)	3' AG TCG CTG GATC	SpeI NheI XbaI
	RC9 (SEQ ID NO:22)	3' AG TCG CTG CTAG	DpnII BglII BamHI BclI BstYI
10	RC10 (SEQ ID NO:23)	3' AG TCG CTG CGCG	KasI
	RC11 (SEQ ID NO:24)	3' AG TCG CTG CCGG	Bsp120 I NotI
15	RC12 (SEQ ID NO:25)	3' AG TCG CTG CATG	Acc56I BsrGI
	RC14 (SEQ ID NO:26)	3' AG TCG CTG AGCT	SalI
	RC15 (SEQ ID NO:27)	3' AG TCG CTG ACGT	Ppu10I ApaI
20	JA24 (SEQ ID NO:28)	5' m-ACC GAC GTC GAC TAT CCA TGA AGA 3'	
	JA1 (SEQ ID NO:29)	3' GT ACT TCT TTAA	Tsp509 I MfeI EcoRI
25	JA5 (SEQ ID NO:30)	3' GT ACT TCT GTAC	NcoI BspHI
	JA6 (SEQ ID NO:31)	3' GT ACT TCT GGCC	XmaI Ngomi BspEI
30	JA7 (SEQ ID NO:32)	3' GT ACT TCT GCGC	BssHII AsCI
	JA8 (SEQ ID NO:33)	3' GT ACT TCT GATC	AvRII NheI XbaI
	JA9 (SEQ ID NO:34)	3' GT ACT TCT CTAG	DpnII BamHI BclI
35	JA10 (SEQ ID NO:35)	3' GT ACT TCT CGCG	KasI

	JA11 (SEQ ID NO:36)	3' GT ACT TCT CCGG	EagI Bsp120 I NotI EaeI
5	JA12 (SEQ ID NO:37)	3' GT ACT TCT CATG	BsiWI Acc65I BsrgI
	JA14 (SEQ ID NO:38)	3' GT ACT TCT AGCT	XhoI Sali
10	JA15 (SEQ ID NO:39)	3' GT ACT TCT ACGT	ApALI
	JA16 (SEQ ID NO:40)	3' GT ACT TCT AATT	AfIII
	JA17 (SEQ ID NO:41)	3' GT ACT TCT AGCA	BssSI
15	JC24 (SEQ ID NO:42)	5' m-ACC GAC GTC GAC TAT CCA TGA AGC 3'	
	JC1 (SEQ ID NO:43)	3' GT ACT TCG TTAA	Tsp509 I EcoRI Apol
20	JC3 (SEQ ID NO:44)	3' GT ACT TCG TCGA	HindII I
	JC5 (SEQ ID NO:45)	3' GT ACT TCG GTAC	BspHI
25	JC6 (SEQ ID NO:46)	3' GT ACT TCG GGCC	AgeI NgomI BspEI SgrAI BsrFI BsaWI
	JC7 (SEQ ID NO:47)	3' GT ACT TCG GCGC	MluI BssHII AscI
30	JC8 (SEQ ID NO:48)	3' GT ACT TCG GTAC	SpeI NheI XbaI
	JC9 (SEQ ID NO:49)	3' GT ACT TCG CTAG	DpnII BglII BamHI BclI BstYI
35			

	JC10 (SEQ ID NO:50)	3' GT ACT TCG CGCG	KasI
5	JC11 (SEQ ID NO:51)	3' GT ACT TCG CCGG	Bsp120 I NotI
	JC12 (SEQ ID NO:52)	3' GT ACT TCG CATG	Acc56I BsrGI
	JC14 (SEQ ID NO:53)	3' GT ACT TCG AGCT	SallI
10	JC15 (SEQ ID NO:54)	3' GT ACT TCG ACGT	Ppu10I ApALI

In the case where one of the primers is conjugated to a capture moiety, Table 11 RE pairs and the corresponding primer/linker combinations that have been tested. This table supplements Table 10. Biotin can be conjugated to primers by 15 using standard phosphoramidite chemistry.

TABLE 11: TESTED RE PAIRS AND BIOTINYLATED ADAPTERS

RE 1	RE 2	Adapter 1	Adapter 2
20		Chose labeled primer JA24 or JC24 to match the linker according to Table 10	Chose biotinylated primer RA24 or RC24 to match the linker according to Table 10
25	BamHI	BspHI	JC9
	BgIII	BspHI	RA5
	BgIII	EcoRI	JC5
	BgIII	HindIII	RC9
30	BgIII	BspEI	JC3
	BgIII	Ncol	RC9
	BspEI	BspYI	JC6
	BspEI	HindIII	RA5
	BspHI	EcoRI	RC9
35	BspHI	HindIII	RC3
	BstYI	EcoRI	JA5
	EcoRI	HindIII	RA1
		JC3	RC9
		JC1	RA1
		JC3	RA1

BAMHI	HindIII	JC9	RC3
BspEI	BspHI	JC6	RA5
BspEI	EcoRI	JC6	RA1
BspHI	BstYI	JA5	RC9
BspHI	NgoMI	JA5	RC6
BstYI	HindIII	JC3	RC9
HindIII	Ncol	JC3	RA5
HindIII	NgoMI	JC3	RC6

10

Tables 12 and 13 list the RE combinations that have been tested in QEA™ experiments on human placental and glandular cDNAs samples. The preferred double digests are those that give more than approximately 50 bands in the range 15 of 100 to 700 bp. Table 12 lists the preferred RE combinations for human cDNA analyses.

TABLE 12: PREFERRED RE COMBINATIONS FOR HUMAN cDNA ANALYSIS

20

Acc56I & HindIII	Acc65I & NgoMI	BamHI & EcoRI
BglIII & HindIII	BglIII & NgoMI	BsiWI & BspHI
BspHI & BstYI	BspHI & NgoMI	BsrGI & EcoRI
EagI & EcoRI	EagI & HindIII	EagI & NcoI
HindIII & NgoMI	NgoMI & NheI	NgoMI & SpeI
BglIII & BspHI	Bsp120I & NcoI	BssHII & NgoMI
EcoRI & HindIII	NgoMI & XbaI	

Table 13 lists other RE combinations tested and 30 that can be used for human cDNA analyses.

35

TABLE 13: OTHER RE COMBINATIONS FOR HUMAN cDNA ANALYSIS

AvrII & NgoMI	BamHI & Bsp120I	BamHI & BspHI
BamHI & NcoI	BclI & BspHI	BclI & NcoI
BglII & BspEI	BglII & EcoRI	BglII & NcoI
BssHII & BsrGI	BstYI & NcoI	BamHI & HindIII
BglII & Bsp120I	BspHI & HindIII	

Tables 14 and 15 list the RE combinations that have
 10 been tested in QEA™ experiments on mouse cDNA samples. The
 preferred double digests are those that give more than
 approximately 50 bands in the range of 100 to 700 bp. Table
 14 lists the preferred RE combinations for mouse cDNA
 analyses.

15

TABLE 14: PREFERRED RE COMBINATIONS FOR
MOUSE cDNA ANALYSIS

Acc56I & HindIII	Acc65I & NgoMI	Ascl & HindIII
AvrII & NgoMI	BamHI & BspHI	BamHI & HindIII
BamHI & NcoI	BclI & NcoI	BglII & BspHI
BglII & HindIII	BglII & NcoI	BglII & NgoMI
Bsp120I & NcoI	Acc65I & BspHI	BspHI & Bsp120I
BspHI & BsrGI	BspHI & EagI	BspHI & NgoMI
BspHI & NotI	BssHII & HindIII	BstYI & HindIII
HindIII & NcoI	HindIII & NgoMI	NcoI & NotI
NgoMI & NheI	NgoMI & SpeI	NgoMI & XbaI
BclI & HindIII		

30 Table 15 lists other RE combinations tested and
 that can be used for mouse cDNA analyses.

35

TABLE 15: OTHER RE COMBINATIONS FOR MOUSE cDNA ANALYSIS

Acc65I & NcoI	BclI & BspHI	BsiWI & BspHI
BsiWI & NcoI	BspHI & HindIII	BsrGI & NcoI
BssHII & NgoMI	BstYI & BspHII	EagI & NcoI
HindIII & MluI		

Table 16 lists the data obtained from various RE combinations using mouse cDNA samples. The number of bands 10 was observed from silver stained acrylamide separation gels.

TABLE 16: MOUSE cDNA RE DIGESTION RESULTS

	RE Combination	Number of Bands
15	Acc65I & HindIII	200
	Acc65I & NgoMI	150
	AscI & HindIII	100
	AvrII & NgoMI	50
20	BamHI & BspHI	200
	BamHI & HindIII	150
	BamHI & NcoI	150
	BclI & BspHI	5
	BclI & HindIII	150
25	BclI & NcoI	50
	BglII & BspHI	50
	BglII & HindIII	150
	BglII & NcoI	50
30	BglII & NgoMI	50
	Bsp120I & NcoI	50
	BspHI & Acc65I	150
	BspHI & Bsp120I	50
	BspHI & BsrGI	200
35	BspHI & EagI	150
	BspHI & HindIII	0

5

10

15

20

RE Combination	Number of Bands
BspHI & NgoMI	150
BspHI & NotI	150
BsrGI & NcoI	10
BssHII & HindIII	100
BssHII & NgoMI	20
BstYI & BspHI	20
BstYI & HindIII	200
EagI & NcoI	10
HindIII & MluI	25
HindIII & NcoI	50
HindIII & NgoMI	150
NcoI & NotI	200
NgoMI & NheI	50
NgoMI & SpeI	200
NgoMI & XbaI	50
TOTAL # BANDS	3490

31 available REs that recognize a 6 bp recognition sequence and generate a 4 bp 5' overhang are: Acc65I, AflIII,
 25 AgeI, ApaLI, ApoI, AscI, AvrI, BamHI, BclI, BglIII, BsiWI,
 Bsp120I, BspEI, BspHI, BsrGI, BssHII, BstYI, EagI, EcoRI,
 HindIII, MfeI, MluI, NcoI, NgoMI, NheI, NotI, Ppu10I, SalI,
 SpeI, XbaI, and XhoI.

All of these enzymes have been tested in QEA™
 30 protocols according to Sec. 6.4.4 with the exception of AflIII. All were useable except for MfeI, Ppu10I, SalI, and XhoI. All the other 26 enzymes have been tested and are usable in the RE implementation of QEA™.

However certain pairs of these enzymes are less
 35 informative due to the fact that they produce identical overhangs, and thus their recognition sequences cannot be distinguished by QEA™ adapters. These pairs are Acc65I and

(BsiWI or BsrGI); AgeI and (BspEI or NcoMI); ApoI and EcoRI; AscI and (BssHII or MluI); AvrI and (NheI, SpeI, or XbaI); BamHI and (BclI, BglII, or BstYI); BclI and (BglII or BstYI); BglII and BstYI; BsiWI and BsrGI; Bsp120I and EagI; BspEI and 5 NcoMI; BspHI and NcoI; BssHII and MluI; NheI and (SpeI or XbaI); and SpeI and XbaI.

Thus 301 RE pairs have been tested and are useable in the RE embodiments of QEA™.

10 6.10.1. PREFERRED SEQ-QEA™ ENZYMES AND ADAPTERS

Table 17 lists exemplary Type IIS REs adaptable to SEQ-QEA™ embodiment and their important characteristics. For each RE, the table lists the recognition sequence on each strand of a dsDNA molecule and the distance in bp from that 15 recognition sequence to the location of strand cutting. Also listed is the net overhang generated.

TABLE 17: SAMPLE TYPE IIS REs

20	Type IIS RE	Recog. Seqs.	Dist. to cutting site (bp)	Overhang (bp)	Comment
	FokI	5'-GGATG CCTAC	9 13	4	
25	HgaI	5'-GACGC CTGCG	5 10	5	
	BbvI	5'-GCAGC CGTCG	8 12	4	
30	BsmFI	5'-GGGAC CCCTG	10 14	4	Lower recognition site specificity
	BspMI	5'-ACCTGC TGGACG	4 8	4	
	SfaNI	5'-GCATC CGTAG	5 9	4	

35 Table 18 lists exemplary primer and linker combinations adaptable to a SEQ-QEA™ method. They satisfy the previously described requirements on primers and linkers.

Except for the indicated differences, they are the same as the primers and linkers of similar names in Table 10. RA24-U and RC24-U have a 5' biotin capture moiety and a uracil release means as indicated, and are adaptable to the same 5 linkers and REs as are RA24 and RC24 of Table 10. RA24-S and RC24-S also have a 5' biotin capture moiety with a AscI recognition site release means as indicated in bold and underlining, and are adaptable to the same linkers and REs as are RA24 and RC24 of Table 10. JA24-K has an internal FokI 10 recognition site as indicated and a 5' FAM label moiety (see Table 19). The FokI recognition site is optimally placed to be used with a RE producing a 4 bp overhang. Linkers KA5, KA6, and KA9 corresponding to the indicated REs function with this primer. JC24-B has an internal BbvI recognition site, a 15 5' FAM label, and functions with linkers BA5 and BA9. The BbvI recognition site is also optimally placed to be used with a RE producing a 4 bp overhang.

TABLE 18: SAMPLE ADAPTERS

20 Series	Adapter: Primer (longer strand) Linker (shorter strand) Notes: 'b' signifies a biotin moiety 'f' signifies a FAM label moiety	RE
RA24-U	5' b-AGC ACT CTC CAG <u>CCU</u> CTC ACC GAA 3' (SEQ ID NO:???)	
25 RA24-S	5' b-AGC ACT <u>CTG GCG CGC</u> CTC ACC GAA 3' (SEQ ID NO:???)	
RC24-U	5' b-AGC ACT CTC CAG <u>CCU</u> CTC ACC GAC 3' (SEQ ID NO:???)	
30 RC24-S	5' b-AGC ACT <u>CTG GCG CGC</u> CTC ACC GAC 3' (SEQ ID NO:???)	
JA24-K	5' f-ACC GAC GTC GAC TAT <u>GGA TGA</u> AGA 3' (SEQ ID NO:???)	FokI (9)

5	KA9 (SEQ ID NO:??)	3' CT ACT TCT CTAG	DpnII BglII BamHI BclI BstYI
10	KA5 (SEQ ID NO:??)	3' CT ACT TCT GTAC	NcoI BspHI
15	KA6 (SEQ ID NO:??)	3' CT ACT TCT GGCC	AgeI NgoMI BspEI SgrAI BsrFI BsaWI
20	JC24-B (SEQ ID NO:??)	5' f-ACC GAC GTC GAC TAT <u>CGC AGC AGA</u> 3'	BbvI (8)
25	BA9 (SEQ ID NO:??)	3' CG TCG TCT CTAG	DpnII BglII BamHI BclI BstYI
30	BA5 (SEQ ID NO:??)	3' CG TCG TCT GTAC	NcoI BspHI

6.11. FLUORESCENT LABELS

Fluorochromes labels that can be used in the methods of the present invention include the classic fluorochromes as well as more specialized fluorochromes. The classic fluorochromes include bimane, ethidium, europium (III) citrate, fluorescein, La Jolla blue, methylcoumarin, nitrobenzofuran, pyrene butyrate, rhodamine, terbium chelate, and tetramethylrhodamine. More specialized fluorochromes are listed in Table 19 along with their suppliers.

TABLE 19: FLORESCENT LABELS

Fluorochrome	Vendor	Absorption Maximum	Emission Maximum
Bodipy 493/503	Molecular Probes	493	503
Cy2	BDS	489	505
Bodipy FL	Molecular Probes	508	516

	Fluorochrome	Vendor	Absorption Maximum	Emission Maximum
5	FTC	Molecular Probes	494	518
	FluorX	BDS	494	520
	FAM	Perkin-Elmer	495	535
	Carboxy-rhodamine	Molecular Probes	519	543
	EITC	Molecular Probes	522	543
10	Bodipy 530/550	Molecular Probes	530	550
	JOE	Perkin-Elmer	525	557
	HEX	Perkin-Elmer	529	560
	Bodipy 542/563	Molecular Probes	542	563
	Cy3	BDS	552	565
15	TRITC	Molecular Probes	547	572
	LRB	Molecular Probes	556	576
	Bodipy LMR	Molecular Probes	545	577
	Tamra	Perkin-Elmer	552	580
	Bodipy 576/589	Molecular Probes	576	589
20	Bodipy 581/591	Molecular Probes	581	591
	Cy3.5	BDS	581	596
	XRITC	Molecular Probes	570	596
	ROX	Perkin-Elmer	550	610
	Texas Red	Molecular Probes	589	615
25	Bodipy TR (618?)	Molecular Probes	596	625
	Cy5	BDS	650	667
	Cy5.5	BDS	678	703
	DdCy5	Beckman	680	710
	Cy7	BDS	443	767
35	D _b Cy7	Beckman	790	820

The suppliers listed in Table 19 are Molecular Probes (Eugene, OR), Biological Detection Systems ("BDS") (Pittsburgh, PA) and Perkin-Elmer (Norwalk, CT).

Means of utilizing these fluorochromes by attaching them to particular nucleotide groups are described in Kricka et al., 1995, Molecular Probing, Blotting, and Sequencing, chap. 1, Academic Press, New York. Preferred methods of attachment are by an amino linker or phosphoramidite chemistry.

10

7. SPECIFIC EMBODIMENTS, CITATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those 15 described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the 20 disclosures of which are incorporated by reference in their entireties.

25

30

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rothberg, Jonathan
Deem, Michael
Simpson, John

5

(ii) TITLE OF INVENTION: Method and Apparatus for Identifying,
Classifying, or Quantifying DNA Sequences in a Sample
Without Sequencing

(iii) NUMBER OF SEQUENCES: 70

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Pennie and Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To be assigned
(B) FILING DATE: 14-June-1995
(C) CLASSIFICATION:

20 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Misrock, S. Leslie
(B) REGISTRATION NUMBER: 18,872
(C) REFERENCE/DOCKET NUMBER: 7934-033-999

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212) 790-9090
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(C) TELEX: 66441 PENNIE

25

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGCACTCTCC AGCCTCTCAC CGAA

24

35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTGGCTTTT AA

12

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
10 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGTGGCTTGT AC

12

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
20 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGTGGCTTGG CC

12

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
30 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTGGCTTGC GC

12

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTGGCTTGA TC

12

(2) INFORMATION FOR SEQ ID NO:7:

10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGTGGCTTCT AG

12

(2) INFORMATION FOR SEQ ID NO:8:

20

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTGGCTTCG CG

12

(2) INFORMATION FOR SEQ ID NO:9:

30

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGTGGCTTCC GG

12

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACTGGCTTCA TG

12

10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGTGGCTTAG CT

12

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGTGGCTTAC GT

12

30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGTGGCTTAA TT

12

(2) INFORMATION FOR SEQ ID NO:14:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

10 AGTGGCTTAG CA

12

(2) INFORMATION FOR SEQ ID NO:15:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

20 AGCACTCTCC AGCCTCTCAC CGAC

24

(2) INFORMATION FOR SEQ ID NO:16:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGTCGCTGTT AA

12

30 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGTCGCTGTC GA

12

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NC:18:

AGTCGCTGGT AC

12

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NC:19:

AGTCGCTGGG CC

12

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

AGTCGCTGGC GC

12

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTCGCTGGA TC

12

(2) INFORMATION FOR SEQ ID NO:22:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGTCGCTGCT AG

12

(2) INFORMATION FOR SEQ ID NO:23:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGTCGCTGCG CG

12

(2) INFORMATION FOR SEQ ID NO:24:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGTCGCTGCC GG

12

(2) INFORMATION FOR SEQ ID NO:25:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AGTCGGCTGCCA TG

12

(2) INFORMATION FOR SEQ ID NO:26:

5

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGTCGGCTGAG CT

12

(2) INFORMATION FOR SEQ ID NO:27:

15

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AGTCGGCTGAC GT

12

(2) INFORMATION FOR SEQ ID NO:28:

25

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ACCGACGTCG ACTATCCATG AAGA

24

(2) INFORMATION FOR SEQ ID NO:29:

35

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GTACTTCTTT AA

12

5 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTACTTCTGT AC

12

(2) INFORMATION FOR SEQ ID NO:31:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GTACTTCTGG CC

12

(2) INFORMATION FOR SEQ ID NO:32:

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GTACTTCTGC GC

12

(2) INFORMATION FOR SEQ ID NO:33:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

5 GTACTTCTGA TC .

12

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTACTTCTCT AG

12

15

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GTACTTCTCG CG

12

25

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTACTTCTCC GG

12

(2) INFORMATION FOR SEQ ID NO:37:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTACTTCTCA TG

12

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GTACTTCTAG CT

12

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GTACTTCTAC GT

12

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GTACTTCTAA TT

12

35 (2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GTACTTCTAG CA

12

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ACCGACGTCG ACTATCCATG AAGC

24

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTACTTCGTT AA

12

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GTACTTCGTC GA

12

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GTACTTCGGT AC

12

(2) INFORMATION FOR SEQ ID NO:46:

10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTACTTCGGG CC

12

(2) INFORMATION FOR SEQ ID NO:47:

20

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GTACTTCGGC GC

12

(2) INFORMATION FOR SEQ ID NO:48:

30

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTACTTCGGT AC

12

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GTACTTCGCT AG

12

10

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GTACTTCGCG CG

12

20

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GTACTTCGCC GG

12

30

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GTACTTCGCA TG

12

(2) INFORMATION FOR SEQ ID NO:53:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

10 GTACTTCGAG CT

12

(2) INFORMATION FOR SEQ ID NO:54:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

20 GTACTTCGAC GT

12

(2) INFORMATION FOR SEQ ID NO:55:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

30 AGCACTCTCC AGCCTCTCAC CGAGCATG

28

(2) INFORMATION FOR SEQ ID NO:56:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

GGCGCGCCTG TAAAACGACG GCCAGTACCG ACGTCGACTA TCCATGAACG

49

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

5

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

AAAACCTGCAG GAAACAGCTA TGACCAGGCAC TCTCCAGCCT CTCACCGA

48

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ACTTCGAAAT TAATACGACT CACTATAGGG ACCGACGTCG ACTATCCATG AAG

53

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

25

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

30

ACTTCGAAAT TAATACGACT CACTATAGGG AGCACTCTCC AGCCTCTCAC CGA

53

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

35

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

AGCACTCTCC AGCCUUCAC CGAA

24

(2) INFORMATION FOR SEQ ID NO:61:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

AGCACTCTGG CGCGCCTCAC CGAA

24

(2) INFORMATION FOR SEQ ID NO:62:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

AGCACTCTCC AGCCUUCAC CGAC

24

(2) INFORMATION FOR SEQ ID NO:63:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

AGCACTCTGG CGCGCCTCAC CGAC

24

(2) INFORMATION FOR SEQ ID NO:64:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

ACCGACGTCG ACTATGGATG AAGA

24

(2) INFORMATION FOR SEQ ID NO:65:

5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

GATCTCTTCA TC

12

(2) INFORMATION FOR SEQ ID NO:66:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CATGTCTTCA TC

12

(2) INFORMATION FOR SEQ ID NO:67:

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CCGGTCTTCA TC

12

(2) INFORMATION FOR SEQ ID NO:68:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

ACCGACGTCG ACTATCGCAG CAGA

24

5 (2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GATCTCTGCT GC

12

(2) INFORMATION FOR SEQ ID NO:70:

15

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CATGTCTGCT GC

12

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WHAT IS CLAIMED IS:

1. A method for identifying, classifying, or quantifying one or more nucleic acids in a sample comprising 5 a plurality of nucleic acids having different nucleotide sequences, said method comprising:

(a) probing said sample with one or more recognition means, each recognition means recognizing a different target nucleotide subsequence or a different set of target 10 nucleotide subsequences;

(b) generating one or more signals from said sample probed by said recognition means, each generated signal arising from a nucleic acid in said sample and comprising a representation of (i) the length between occurrences of 15 target subsequences in said nucleic acid, and (ii) the identities of said target subsequences in said nucleic acid or the identities of said sets of target subsequences among which are included the target subsequences in said nucleic acid; and

20 (c) searching a nucleotide sequence database to determine sequences that match or the absence of any sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences of nucleic acids that may be present in the sample, a 25 sequence from said database matching a generated signal when the sequence from said database has both (i) the same length between occurrences of target subsequences as is represented by the generated signal, and (ii) the same target subsequences as are represented by the generated signal, or 30 target subsequences that are members of the same sets of target subsequences represented by the generated signal, whereby said one or more nucleic acids in said sample are identified, classified, or quantified.

35 2. The method of claim 1 wherein each recognition means recognizes one target subsequence, and wherein a sequence from said database matches a generated signal when

the sequence from said database has both the same length between occurrences of target subsequences as is represented by the generated signal and the same target subsequences as represented by the generated signal.

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3. The method of claim 1 wherein each recognition means recognizes a set of target subsequences, and wherein a sequence from said database matches a generated signal when the sequence from said database has both the same length 10 between occurrences of target subsequences as is represented by the generated signal, and the target subsequences are members of the sets of target subsequences represented by the generated signal.

15 4. The method of claim 1 further comprising dividing said sample of nucleic acids into a plurality of portions and performing the steps of claim 1 individually on a plurality of said portions, wherein a different one or more recognition means are used with each portion.

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5. The method of claim 1 wherein the quantitative abundance of nucleic acids containing said nucleotide sequences in the sample is determined from the quantitative level of the one or more signals determined to match said 25 sequences.

6. The method of claim 1 wherein said plurality of nucleic acids are DNA.

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7. The method of claim 6 wherein the DNA is cDNA.

8. The method of claim 7 wherein the cDNA is prepared from a plant, a single celled animal, a multicellular animal, a bacterium, a virus, a fungus, or a yeast.

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9. The method of claim 8 wherein said database comprises substantially all the known expressed sequences of

1. said plant, single celled animal, multicellular animal, bacterium, virus, fungus, or yeast.

10. The method of claim 7 wherein the cDNA is of total 5 cellular RNA or total cellular poly(A) RNA.

11. The method of claim 6 wherein the recognition means are one or more restriction endonucleases whose recognition sites are said target subsequences, and wherein
10 the step of probing comprises digesting said sample with said one or more restriction endonucleases into fragments and ligating double stranded adapter DNA molecules to said fragments to produce ligated fragments, each said adapter DNA molecule comprising (i) a shorter stand having no 15 5' terminal phosphates and consisting of a first and second portion, said first portion at the 5' end of the shorter strand and being complementary to the overhang produced by one of said restriction endonucleases, and (ii) a longer strand having a 3' end subsequence complementary to said 20 second portion of the shorter strand; and wherein
the step of generating further comprises melting the shorter strand from the ligated fragments, contacting the ligated fragments with a DNA polymerase, extending the ligated fragments by synthesis with the DNA polymerase to 25 produce blunt-ended double stranded DNA fragments, and amplifying the blunt-ended fragments by a method comprising contacting the blunt-ended fragments with the DNA polymerase and primer oligodeoxynucleotides, said primer oligodeoxynucleotides comprising the longer adapter strand, 30 and said contacting being at a temperature not greater than the melting temperature of the primer oligodeoxynucleotide from a strand of the blunt-ended fragments complementary to the primer oligodeoxynucleotide and not less than the melting temperature of the shorter strand of the adapter nucleic acid 35 from the blunt-ended fragments.

12. The method of claim 6 wherein the recognition means are one or more restriction endonucleases whose recognition sites are said target subsequences, and wherein the step of probing further comprises digesting the sample 5 with said one or more restriction endonucleases.

13. The method of claim 12 further comprising:
(a) identifying a fragment of a nucleic acid in the sample which generates said one or more signals; and
10 (b) recovering said fragment.

14. The method of claim 13 wherein the signals generated by said recovered fragment do not match a sequence in said nucleotide sequence database.

15 15. The method of claim 13 which further comprises using at least a hybridizable portion of said fragment as a hybridization probe to bind to a nucleic acid that can generate said fragment upon digestion by said one or more 20 restriction endonucleases.

16. The method of claim 12 wherein the step of generating further comprises after said digesting: removing from the sample both nucleic acids which have not been 25 digested and nucleic acid fragments resulting from digestion at only a single terminus of the fragments.

17. The method of claim 16 wherein prior to digesting, the nucleic acids in the sample are each bound at one 30 terminus to a biotin molecule, and said removing is carried out by a method which comprises contacting the nucleic acids in the sample with streptavidin or avidin affixed to a solid support.

35 18. The method of claim 16 wherein prior to digesting, the nucleic acids in the sample are each bound at one terminus to a hapten molecule, and said removing is carried

out by a method which comprises contacting the nucleic acids in the sample with an anti-hapten antibody affixed to a solid support.

5 19. The method of claim 12 wherein said digesting with said one or more restriction endonucleases leaves single-stranded nucleotide overhangs on the digested ends.

10 20. The method of claim 19 wherein the step of probing further comprises hybridizing double-stranded adapter nucleic acids with the digested sample fragments, each said adapter nucleic acid having an end complementary to said overhang generated by a particular one of the one or more restriction endonucleases, and ligating with a ligase a strand of said 15 adapter nucleic acids to the 5' end of a strand of the digested sample fragments to form ligated nucleic acid fragments.

20 21. The method of claim 20 wherein said digesting with said one or more restriction endonucleases and said ligating are carried out in the same reaction medium.

25 22. The method of claim 21 wherein said digesting and said ligating comprises incubating said reaction medium at a first temperature and then at a second temperature, wherein said one or more restriction endonucleases are more active at the first temperature than the second temperature and said ligase is more active at the second temperature than the first temperature.

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23. The method of claim 22 wherein said incubating at said first temperature and said incubating at said second temperature are performed repetitively.

35 24. The method of claim 20 wherein the step of probing further comprises prior to said digesting: removing terminal

phosphates from DNA in said sample by incubation with an alkaline phosphatase.

25. The method of claim 24 wherein said alkaline phosphatase is heat labile and is heat inactivated prior to said digesting.

26. The method of claim 20 wherein said generating step comprises amplifying the ligated nucleic acid fragments.

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27. The method of claim 26 wherein said amplifying is carried out by use of a nucleic acid polymerase and primer nucleic acid strands, said primer nucleic acid strands being capable of priming nucleic acid synthesis by said polymerase.

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28. The method of claim 27 wherein the primer nucleic acid strands have a G+C content of between 40% and 60%.

29. The method of claim 27 wherein each said adapter nucleic acid has a shorter strand and a longer strand, the longer strand being ligated to the digested sample fragments, and said generating step comprises prior to said amplifying step the melting of the shorter strand from the ligated fragments, contacting the ligated fragments with a DNA polymerase, extending the ligated fragments by synthesis with the DNA polymerase to produce blunt-ended double stranded DNA fragments, and wherein the primer nucleic acid strands comprise a hybridizable portion of the sequence of said longer strands, each different primer nucleic acid strand priming amplification only of blunt ended double stranded DNA fragments that are produced after digestion by a particular restriction endonuclease.

30. The method of claim 27 wherein each said adapter nucleic acid has a shorter strand and a longer strand, the longer strand being ligated to the digested sample fragments, and said generating step comprises prior to said amplifying

step the melting of the shorter strand from the ligated fragments, contacting the ligated fragments with a DNA polymerase, extending the ligated fragments by synthesis with the DNA polymerase to produce blunt-ended double stranded DNA 5 fragments, and wherein the primer nucleic acid strands comprise the sequence of said longer strands, each different primer nucleic acid strand priming amplification only of blunt ended double stranded DNA fragments that are produced after digestion by a particular restriction endonuclease.

10

31. The method of claim 30 wherein during said amplifying step the primer nucleic acid strands are annealed to the ligated nucleic acid fragments at a temperature that is less than the melting temperature of the primer nucleic 15 acid strands from strands complementary to the primer nucleic acid strands but greater than the melting temperature of the shorter adapter strands from said blunt-ended fragments.

32. The method of claim 30 wherein the primer nucleic 20 acid strands comprise primers, each primer specific for a particular restriction endonuclease, and further comprising at the 3' end of and contiguous with the longer strand sequence, the portion of the restriction endonuclease recognition site remaining on a nucleic acid fragment 25 terminus after digestion by the restriction endonuclease.

33. The method of claim 32 wherein each said primer specific for a particular restriction endonuclease further comprises at its 3' end one or more nucleotides 3' to and 30 contiguous with the remaining portion of the restriction endonuclease recognition site, whereby the ligated nucleic acid fragment amplified is that comprising said remaining portion of said restriction endonuclease recognition site contiguous to said one or more additional nucleotides.

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34. The method of claim 33 wherein said specific primers are detectably labeled, such that said primers

comprising a particular said one or more additional nucleotides can be distinguishably detected from said primers comprising a different said one or more additional nucleotides.

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35. The method of claim 6 wherein the recognition means are oligomers of nucleotides, nucleotide-mimics, or a combination of nucleotides and nucleotide-mimics, which are specifically hybridizable with the target subsequences.

10

36. The method of claim 35 wherein the step of generating comprises amplifying with a nucleic acid polymerase and with primers comprising said oligomers, whereby fragments of nucleic acids in the sample between 15 hybridized oligomers are amplified.

37. The method of claim 36 further comprising:
(a) identifying a fragment of a nucleic acid in the sample which generates said one or more signals; and
20 (b) recovering said fragment.

38. The method of claim 37 wherein the signals generated by said recovered fragment do not match a sequence in said nucleotide database.

25

39. The method of claim 37 which further comprises using at least a hybridizable portion of said fragment as a hybridization probe to bind to a nucleic acid that can generate said fragment upon amplification with said nucleic acid polymerase and said one or more primers.

40. The method of claim 1 wherein said signals further comprise a representation of whether an additional target subsequence is present on said nucleic acid in the sample 35 between said occurrences of target subsequences.

41. The method of claim 40 wherein said additional target subsequence is recognized by a method comprising contacting nucleic acids in the sample with oligomers of nucleotides, nucleotide-mimics, or mixed nucleotides and 5 nucleotide-mimics, which are hybridizable with said additional target subsequence.

42. The method of claim 1 wherein the step of generating comprises suppressing said signals when an 10 additional target subsequence is present on said nucleic acid in the sample between said occurrences of target subsequences.

43. The method of claim 42 wherein the step of 15 generating comprises amplifying nucleic acids in the sample, and wherein said additional target subsequence is recognized by a method comprising contacting nucleic acids in the sample with (a) oligomers of nucleotides, nucleotide-mimics, or mixed nucleotides and nucleotide-mimics, which hybridize with 20 said additional target subsequence and disrupt the amplifying step; or (b) restriction endonucleases which have said additional target subsequence as a recognition site and digest the nucleic acids in the sample at the recognition site.

25

44. The method of claim 12 or 36 wherein the step of generating further comprises separating nucleic acid fragments by length.

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45. The method of claim 44 wherein the step of generating further comprises detecting said separated nucleic acid fragments.

35

46. The method of claim 45 wherein the quantitative abundance of a nucleic acid comprising a particular nucleotide sequence in the sample is determined from the quantitative level of the one or more signals generated by

said nucleic acid that are determined to match said particular nucleotide sequence.

47. The method of claim 45 wherein said detecting is 5 carried out by a method comprising staining said fragments with silver, labeling said fragments with a DNA intercalating dye, or detecting light emission from a fluorochrome label on said fragments.

10 48. The method of claim 45 wherein said representation of the length between occurrences of target subsequences is the length of fragments determined by said separating and detecting steps.

15 49. The method of claim 45 wherein said separating is carried out by use of liquid chromatography or mass spectrometry.

50. The method of claim 45 wherein said separating is 20 carried out by use of electrophoresis.

51. The method of claim 50 wherein said electrophoresis is carried out in a slab gel or capillary configuration using a denaturing or non-denaturing medium.

25 52. The method of claim 1 wherein a predetermined one or more nucleotide sequences in said database are of interest, and wherein the target subsequences are such that said sequences of interest generate at least one signal that 30 is not generated by other nucleotide sequences in said database.

53. The method of claim 52 wherein the nucleotide sequences of interest are a majority of the sequences in said 35 database.

54. The method of claim 1 wherein the target subsequences have a probability of occurrence in the nucleotide sequences in said database of from approximately 0.01 to approximately 0.30.

5

55. The method of claim 1 wherein the target subsequences are such that nucleotide sequences in said database contain on average a sufficient number of occurrences of target subsequences in order to on average 10 generate a signal that is not generated by any other nucleotide sequence in said database.

56. The method of claim 55 wherein the number of pairs of target subsequences present on average in a nucleotide sequence in said database is no less than 3, and wherein the 15 average number of signals generated from nucleotide sequences in said database is such that the average difference between lengths represented by the generated signals is greater than or equal to 1 nucleotide.

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57. The method of claim 55 wherein the target subsequences have a probability of occurrence, p, approximately given by the solution of

25

$$\frac{R(R+1)p^2}{2} = A$$

and

30

$$\frac{L}{Np^2} = B$$

35

wherein N = the number of different nucleotide sequences in said database; L = the average length of said different nucleotide sequences in said database; R = the number of recognition means; A = the number of pairs of target subsequences present on average in said different nucleotide sequences in said database; and B = the average difference

between lengths represented by the signals generated from the sequences in said database.

58. The method of claim 57 wherein A is greater than 5 or equal to 3.

59. The method of claim 57 wherein B is greater than or equal to 1.

10 60. The method of claim 1 wherein the target subsequences are selected according to the further steps comprising:

(a) determining a pattern of signals that can be generated and the sequences capable of generating each such 15 signal by simulating the steps of probing and generating applied to sequences in said database of nucleotide sequences;

(b) ascertaining the value of said determined pattern according to an information measure; and

20 (c) choosing the target subsequences in order to generate a new pattern that optimizes the information measure.

61. The method of claim 60 wherein said choosing step 25 selects target subsequences which comprise the recognition sites of the one or more restriction endonucleases.

62. The method of claim 60 wherein said choosing step selects target subsequences which comprise the recognition 30 sites of the one or more restriction endonucleases contiguous with one or more additional nucleotides.

63. The method of claim 60 wherein a predetermined one or more of the nucleotide sequences present in said database 35 of nucleotide sequences are of interest, and the information measure optimized is the number of such said sequences of interest which generate at least one signal that is not

generated by any other nucleotide sequence present in said database.

64. The method of claim 63 wherein said nucleotide sequences of interest are a majority of the nucleotide sequences present in said database.

65. The method of claim 60 wherein said choosing step is by exhaustive search of all combinations of target subsequences of length less than approximately 10.

66. The method of claim 60 wherein said step of choosing target subsequences is by a method comprising simulated annealing.

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67. The method of claim 1 wherein the step of searching further comprises:

(a) determining a pattern of signals that can be generated and the sequences capable of generating each such signal by simulating the steps of probing and generating applied to each sequence in said database of nucleotide sequences; and

(b) finding the one or more nucleotide sequences in said database that are able to generate said one or more generated signals by finding in said pattern those signals that comprise a representation of (i) the same lengths between occurrences of target subsequences as is represented by the generated signal, and (ii) the same target subsequences as are represented by the generated signal, or target subsequences that are members of the same sets of target subsequences represented by the generated signal.

68. The method of claim 60 or 67 wherein the step of determining further comprises:

35 (a) searching for occurrences of said target subsequences or sets of target subsequences in nucleotide sequences in said database of nucleotide sequences;

(b) finding the lengths between occurrences of said target subsequences or sets of target subsequences in the nucleotide sequences of said database; and

5 (c) forming the pattern of signals that can be generated from the sequences of said database in which the target subsequences were found to occur.

69. The method of claim 20 wherein said restriction endonucleases generate 5' overhangs at the terminus of 10 digested fragments and wherein each double stranded adapter nucleic acid comprises:

(a) a shorter nucleic acid strand consisting of a first and second contiguous portion, said first portion being a 5' end subsequence complementary to the overhang produced 15 by one of said restriction endonucleases; and

(b) a longer nucleic acid strand having a 3' end subsequence complementary to said second portion of the shorter strand.

20 70. The method of claim 69 wherein said shorter strand has a melting temperature from a complementary strand of less than approximately 68 °C, and has no terminal phosphate.

71. The method of claim 70 wherein said shorter strand 25 is approximately 12 nucleotides long.

72. The method of claim 69 wherein said longer strand has a melting temperature from a complementary strand of greater than approximately 68 °C, is not complementary to any 30 nucleotide sequence in said database, and has no terminal phosphate.

73. The method of claim 72 wherein said ligated nucleic acid fragments do not contain a recognition site for 35 any of said restriction endonucleases.

74. The method of claim 72 wherein said one or more restriction endonucleases are heat inactivated before said ligating.

5 75. The method of claim 72 wherein said longer strand is approximately 24 nucleotides long and has a G+C content between 40% and 60%.

10 76. The method of claim 20 wherein said restriction endonucleases generate 3' overhangs at the terminus of the digested fragments, and wherein each double stranded adapter nucleic acid comprises:

15 (a) a longer nucleic acid strand consisting of a first and second contiguous portion, said first portion being a 3' end subsequence complementary to the overhang produced by one of said restriction endonucleases; and

20 (b) a shorter nucleic acid strand complementary to the 3' end of said second portion of the longer nucleic acid stand.

20

77. The method of claim 76 wherein said shorter strand has a melting temperature from said longer strand of less than approximately 68 °C, and has no terminal phosphates.

25 78. The method of claim 77 wherein said shorter strand is 12 base pairs long.

79. The method of claim 76 wherein said longer strand has a melting temperature from a complementary strand of 30 greater than approximately 68 °C, is not complementary to any nucleotide sequence in said database, has no terminal phosphate, and wherein said ligated nucleic acid fragments do not contain a recognition site for any of said restriction endonucleases.

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80. The method of claim 79 wherein said longer strand is 24 base pairs long and has a G+C content between 40% and 60%.

5 81. A method for identifying or classifying a nucleic acid comprising:

(a) probing said nucleic acid with a plurality of recognition means, each recognition means recognizing a target nucleotide subsequence or a set of target nucleotide 10 subsequences, in order to generate a set of signals, each signal representing whether said target subsequence or one of said set of target subsequences is present or absent in said nucleic acid; and

(b) searching a nucleotide sequence database, said 15 database comprising a plurality of known nucleotide sequences of nucleic acids that may be present in the sample, for sequences matching said generated set of signals, a sequence from said database matching a set of signals when the sequence from said database (i) comprises the same target 20 subsequences as are represented as present, or comprises target subsequences that are members of the sets of target subsequences represented as present by the generated sets of signals, and (ii) does not comprise the target subsequences represented as absent or that are members of the sets of 25 target subsequences represented as absent by the generated sets of signals,

whereby the nucleic acid is identified or classified.

82. The method of claim 81 wherein the set of signals 30 are represented by a hash code which is a binary number.

83. The method of claim 81 wherein the step of probing generates quantitative signals of the numbers of occurrences of said target subsequences or of members of said set of 35 target subsequences in said nucleic acid.

84. The method of claim 83 wherein a sequence matches said generated set of signals when the sequence from said database comprises the same target subsequences with the same number of occurrences in said sequence as in the quantitative 5 signals and does not comprise the target subsequences represented as absent or target subsequences within the sets of target subsequences represented as absent.

85. The method of claim 81 wherein said plurality of 10 nucleic acids are DNA.

86. The method of claim 85 wherein the recognition means are detectably labeled oligomers of nucleotides, nucleotide-mimics, or combinations of nucleotides and 15 nucleotide-mimics, and the step of probing comprises hybridizing said nucleic acid with said oligomers.

87. The method of claim 86 wherein said detectably labeled oligomers are detected by a method comprising 20 detecting light emission from a fluorochrome label on said oligomers, or arranging said labeled oligomers to cause light to scatter from a light pipe and detecting said scattering.

88. The method of claim 86 wherein the recognition 25 means are oligomers of peptido-nucleic acids.

89. The method of claim 86 wherein the recognition means are DNA oligomers, DNA oligomers comprising universal nucleotides, or sets of partially degenerate DNA oligomers.

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90. The method of claim 85 wherein the step of searching further comprises:

(a) determining a pattern of sets of signals of the presence or absence of said target subsequences or said sets 35 of target subsequences that can be generated and the sequences capable of generating each set of signals in said

pattern by simulating the step of probing as applied to each sequence in said database of nucleotide sequences; and

(b) finding one or more nucleotide sequences that are capable of generating said generated set of signals by 5 finding in said pattern those sets that match said generated set, where a set of signals from said pattern matches a generated set of signals when the set from said pattern (i) represents as present the same target subsequences as are represented as present or target subsequences that are 10 members of the sets of target subsequences represented as present by the generated sets of signals and (ii) represents as absent the target subsequences represented as absent or that are members of the sets of target subsequences represented as absent by the generated sets of signals.

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91. The method of claim 85 wherein the target subsequences are selected according to the further steps comprising:

(a) determining (i) a pattern of sets of signals 20 representing the presence or absence of said target subsequences or of said sets of target subsequences that can be generated, and (ii) the sequences capable of generating each set of signals in said pattern by simulating the step of probing as applied to each sequence in said database of 25 nucleotide sequences;

(b) ascertaining the value of said pattern generated according to an information measure; and

(c) choosing the target subsequences in order to generate a new pattern that optimizes the information 30 measure.

92. The method of claim 91 wherein the information measure is the number of sets of signals in the pattern which are capable of being generated by one or more sequences in 35 said database.

93. The method of claim 91 wherein the information measure is the number of sets of signals in the pattern which are capable of being generated by only one sequence in said database.

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94. The method of claim 91 wherein said choosing step is by a method comprising exhaustive search of all combination of target subsequences of length less than approximately 10.

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95. The method of claim 91 wherein said choosing step is by a method comprising simulated annealing.

96. The method of claim 90 or 91 wherein the step of 15 determining by simulating further comprises:

(a) searching for the presence or absence of said target subsequences or sets of target subsequences in each nucleotide sequence in said database of nucleotide sequences; and

20 (b) forming the pattern of sets of signals that can be generated from said sequences in said database.

97. The method of claim 96 where the step of searching is carried out by a string search.

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98. The method of claim 96 wherein the step of searching comprises counting the number of occurrences of said target subsequences in each nucleotide sequence.

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99. The method of claim 81 wherein the target subsequences have a probability of occurrence in a nucleotide sequence in said database of nucleotide sequences of from 0.01 to 0.6.

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100. The method of claim 99 wherein the target subsequences are such that the presence of one target subsequence in a nucleotide sequence in said database of

nucleotide sequences is substantially independent of the presence of any other target subsequence in the nucleotide sequence.

5 101. The method of claim 99 wherein fewer than approximately 50 target subsequences are selected.

102. A programmable apparatus for analyzing signals comprising:

10 (a) an inputting device for inputting one or more actual signals generated by probing a sample comprising a plurality of nucleic acids with recognition means, each recognition means recognizing a target nucleotide subsequence or a set of target nucleotide subsequences, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a nucleic acid of said sample, and (ii) the identities of said target subsequences in said nucleic acid, or the identities of said sets of target subsequences among which is included the 20 target subsequences in said nucleic acid;

25 (b) a searching device operatively coupled to said accepting device for searching a sequence in a nucleotide sequence database for occurrences of said target subsequences or target subsequences that are members of said sets of target subsequences, and for the length between such occurrences, said database comprising a plurality of known nucleotide sequences that may be present in said sample;

30 (c) a comparing device operatively coupled to said accepting device and to said searching device for finding a match between said one or more actual signals and a sequence in said database, said one or more actual signals matching a sequence from said database when the sequence from said database has both (i) the same length between occurrences of target subsequences as is represented by said one or more 35 actual signals, and (ii) the same target subsequences as are represented by said one or more actual signals, or target subsequences that are members of the sets of target

subsequences represented by said one or more actual signals; and

(d) a control device operatively coupled to said comparing device for causing said comparing to be done for 5 sequences in the database and for outputting those database sequences that match said one or more actual signals.

103. The programmable apparatus of claim 102 wherein said searching device searches for said target subsequences 10 or a set of target nucleotide subsequences in said database sequences by performing a string comparison of the nucleotides in said subsequences with those in said database sequence.

15 104. The programmable apparatus of claim 102 wherein said control device further comprises causing said searching device to search all sequences in said database in order to determine a pattern of signals that can be generated by probing said sample with said recognition means, and wherein 20 said control device further causes said comparing device to find any matches between said one or more actual signals and said pattern of signals, said one or more actual signals matching a signal in said pattern of signals when the signal from said pattern represents (i) the same length between 25 occurrences of target subsequences as is represented by said one or more actual signals, and (ii) the same target subsequences as are represented by said one or more actual signals, or target subsequences that are members of the sets of target subsequences represented by said one or more actual 30 signals.

105. The programmable apparatus of claim 102 wherein said sample of nucleic acids comprises cDNA of RNA of a cell or tissue type, and said database comprises DNA sequences 35 that are likely to be expressed by said cell or tissue type.

106. A computer readable memory that can be used to direct a programmable apparatus to function for analyzing signals according to steps comprising:

(a) inputting one or more actual signals generated by probing a sample comprising a plurality of nucleic acids with recognition means, each recognition means recognizing a target nucleotide subsequence or a set of target nucleotide subsequences, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a nucleic acid of said sample, and (ii) the identities of said target subsequences in said nucleic acid, or the identities of said sets of target subsequences among which is included the target subsequences in said nucleic acid;

(b) searching a sequence in a nucleotide sequence database for occurrences of said target subsequences or target subsequences that are members of said sets of target subsequences, and for the length between such occurrences, said database comprising a plurality of known nucleotide sequences that may be present in said sample;

(c) matching said one or more actual signals and a sequence in said database when the sequence in said database has both (i) the same length between occurrences of target subsequences as is represented by said one or more actual signals and (ii) the same target subsequences as are represented by said one or more actual signals, or target subsequences that are members of the sets of target subsequences as are represented by said one or more actual signals; and

(d) repetitively performing said searching and matching steps for the majority of sequences in the database and outputting those database sequences that match said one or more actual signals.

107. A programmable apparatus for selecting target subsequences comprising:

(a) an initial selection device for selecting initial target subsequences or initial sets of target subsequences;

(b) a first control device;

(c) a search device operatively coupled to said initial selection device and to said first control device (i) for searching sequences in a nucleotide sequence database for 5 occurrences of said initial target subsequences or occurrences of target subsequences that are members of said initial sets of target subsequences and for the length between such occurrences, and (ii) for determining an initial pattern of signals that can be generated from said selected 10 initial target subsequences or said initial sets of target subsequences, said database comprising a plurality of known nucleotide sequences, said signals comprising a representation of (i) the length between said occurrences in a sequence in said database, and (ii) the identities of said 15 initial target subsequences that occur in said sequence in said database, or the identities of target subsequences that are members of the initial sets of target subsequences that occur in said sequence in said database; and

(d) an ascertaining device operatively coupled to said 20 searching device and to said first control device for ascertaining the value of said determined initial pattern according to an information measure; and wherein

 said first control device causes further target subsequences to be selected and causes the search device to 25 determine a further pattern of signals and the ascertaining device to ascertain a further value of said information measure and accepts the further target subsequences when said further pattern optimizes said further value of said information measure.

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108. The programmable apparatus of claim 107 wherein a predetermined one or more of the sequences in said database are of interest, and wherein said ascertaining device ascertains the value of an information measure by counting 35 the number of such sequences of interest which generate in said determined pattern at least one signal that is not generated by any other sequence in said database.

109. The programmable apparatus of claim 108 wherein said one or more of the sequences of interest comprise substantially all the sequences in said database.

5 110. The programmable apparatus of claim 107 wherein said first control device optimizes the value of said information measure according to a method of exhaustive search, wherein said first control device selects further target subsequences of length less than approximately 10 and 10 accepts the further target subsequences if said further value of said information measure is greater than the previous value.

111. The programmable apparatus of claim 107 wherein 15 said first control device optimizes the value of said information measure according to a method comprising simulated annealing, wherein said first control device repeatedly selects further target subsequences and accepts the further target subsequences if said further value of said 20 information measure is not decreased by greater than a probabilistic factor dependent on a simulated-temperature, and wherein said programmable apparatus further comprises a second control device operatively coupled to said first control device for decreasing said simulated-temperature as 25 said first control device selects further target subsequences.

112. The programmable apparatus of claim 111 wherein said probabilistic factor is an exponential function of the 30 negative of the decrease in the information measure divided by said simulated-temperature.

113. The programmable apparatus of claim 107 wherein said database comprises a majority of known DNA sequences 35 that are likely to be expressed in one or more cell types.

114. A computer readable memory that can be used to direct a programmable apparatus to function for selecting target subsequences according to steps comprising:

(a) selecting initial target subsequences or initial 5 sets of target subsequences;

(b) searching a sequence in a nucleotide sequence database for occurrences of said initial target subsequences or occurrences of target subsequences that are members of said initial sets of target subsequences and for the length 10 between such occurrences, said database comprising a plurality of known nucleotide sequences that may be present in said sample;

(c) determining an initial pattern of signals that can be generated from said selected initial target subsequences 15 or said initial sets of target subsequences, said signals comprising a representation of (i) the length between said occurrences in a sequence in said database, and (ii) the identities of said initial target subsequences that occur in said sequence in said database, or the identities of target 20 subsequences that are members of the initial sets of target subsequences that occur in said sequence in said database; and

(d) ascertaining the value of said determined initial pattern according to an information measure; and

25 (e) repetitively performing said selecting, searching, determining, and ascertaining steps to determine a further pattern of signals and a further value of said information measure, and accepting the further target subsequences when said further pattern optimizes said further value of said 30 information measure.

115. A programmable apparatus for displaying data comprising:

(a) a selecting device for selecting target 35 subsequences or sets of target subsequences, such that recognition means for recognizing said target subsequences or said sets of target subsequences can be used to generate

signals by probing a sample comprising a plurality of nucleic acids, said signals comprising a representation of (i) the length between occurrences of said target subsequences in a nucleic acid of said sample and (ii) the identities of said 5 target subsequences in said nucleic acid or the identities of said sets of target subsequences among which are included the target subsequences in said nucleic acid;

(b) an inputting device for inputting one or more actual signals generated by probing said sample with said 10 recognition means;

(c) an analyzing device for analyzing signals operatively coupled to said selecting and inputting devices that determines which sequences in a nucleotide sequence database can generate said actual signals when subject to 15 said recognition means, said database comprising a plurality of known nucleotide sequences that may be present in said sample;

(d) an input/output device operatively coupled to said selecting, inputting, and analyzing devices that inputs user 20 requests and controls the selecting device to select target subsequences or sets of target subsequences, controls the inputting device to accept actual signals, controls the analyzing device to find the sequences in said database that can generate said actual signals, and displays output 25 comprising said actual signals and said sequences in said database that can generate said actual signals.

116. The programmable apparatus of 115 wherein said sample is a cDNA sample prepared from a tissue specimen, and 30 the apparatus further comprises a storage device operatively coupled to the input/output device for storing indications of the origin of said tissue specimen and information concerning said tissue specimen,

and wherein said indications can be displayed upon user 35 input.

117. The programmable apparatus of 116 wherein the indications and information concerning said tissue specimen comprises histological information comprising tissue images.

5 118. The programmable apparatus of claim 115 further comprising:

(a) one or more instrument devices for probing said sample with said recognition means and for generating said actual signals; and

10 (b) a control device operatively coupled to said one or more instrument devices and to said input/output device for controlling the operation of said instrument devices,

wherein said user can input control commands for control of said instrument devices and receive output concerning the 15 status of said instrument devices.

119. The programmable apparatus of 118 wherein the one or more instrument devices are capable of automatic operation, whereby the probing and generating can be 20 performed without manual intervention.

120. The programmable apparatus of claim 115 wherein one or more of said selecting, inputting, analyzing, and input/output devices are physically collocated with each 25 other.

121. The programmable apparatus of claim 115 wherein one or more of said selecting, inputting, analyzing, and input/output devices are physically spaced apart from each 30 other and are connected by a communication medium for exchanges of commands and information.

122. A computer readable memory that can be used to direct a programmable apparatus to function for displaying 35 data according to steps comprising:

(a) selecting target subsequences or sets of target subsequences, such that recognition means for recognizing

said target subsequences or said sets of target subsequences can be used to generate signals by probing a sample comprising a plurality of nucleic acids, said signals comprising a representation of (i) the length between 5 occurrences of said target subsequences in a nucleic acid of said sample and (ii) the identities of said target subsequences in said nucleic acid or the identities of said sets of target subsequences among which are included the target subsequences in said nucleic acid;

10 (b) inputting one or more actual signals generated by probing said sample with said recognition means;

(c) analyzing said one or more actual signals to determine which sequences in a nucleotide sequence database can generate said actual signals when subject to said 15 recognition means, said database comprising a plurality of known nucleotide sequences that may be present in said sample; and

(d) inputting user requests to control said selecting step to select target subsequences or sets of target 20 subsequences, said inputting step to input actual signals, and said analyzing step to find the sequences in said database that can generate said actual signals, and outputting in response to further user requests information comprising said actual signals and said sequences in said 25 database that can generate said actual signals.

123. A method for identifying, classifying, or quantifying DNA molecules in a sample of DNA molecules having a plurality of different nucleotide sequences, the method 30 comprising the steps of:

(a) digesting said sample with one or more restriction endonucleases, each said restriction endonuclease recognizing a subsequence recognition site and digesting DNA at said recognition site to produce fragments with 5' overhangs;

35 (b) contacting said fragments with shorter and longer oligodeoxynucleotides, each said shorter oligodeoxynucleotide hybridizable with a said 5' overhang and having no terminal

phosphates, each said longer oligodeoxynucleotide hybridizable with a said shorter oligodeoxynucleotide;

5 (c) ligating said longer oligodeoxynucleotides to said 5' overhangs on said DNA fragments to produce ligated DNA 5 fragments;

(d) extending said ligated DNA fragments by synthesis with a DNA polymerase to produce blunt-ended double stranded DNA fragments;

(e) amplifying said blunt-ended double stranded DNA 10 fragments by a method comprising contacting said DNA fragments with a DNA polymerase and primer oligodeoxynucleotides, each said primer oligodeoxynucleotide having a sequence comprising that of one of the longer oligodeoxynucleotides;

15 (f) determining the length of the amplified DNA fragments; and

(g) searching a DNA sequence database, said database comprising a plurality of known DNA sequences that may be present in the sample, for sequences matching one or more of 20 said fragments of determined length, a sequence from said database matching a fragment of determined length when the sequence from said database comprises recognition sites of said one or more restriction endonucleases spaced apart by the determined length,

25 whereby DNA molecules in said sample are identified, classified, or quantified.

124. The method of claim 123 wherein the sequence of each primer oligodeoxynucleotide further comprises 3' to and 30 contiguous with the sequence of the longer oligodeoxynucleotide the portion of the recognition site of said one or more restriction endonucleases remaining on a DNA fragment terminus after digestion, said remaining portion being 5' to and contiguous with one or more additional 35 nucleotides, and wherein a sequence from said database matches a fragment of determined length when the sequence from said database comprises subsequences that are the

recognition sites of said one or more restriction endonucleases contiguous with said one or more additional nucleotides and when the subsequences are spaced apart by the determined length.

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125. The method of claim 123 wherein said determining step further comprises detecting the amplified DNA fragments by a method comprising staining said fragments with silver.

10 126. The method of claim 123 wherein said longer oligodeoxynucleotides are detectably labeled, wherein the determining step further comprises detection of said detectable labels, and wherein a sequence from said database matches a fragment of determined length when the sequence 15 from said database comprises recognition sites of the one or more restriction endonucleases, said recognition sites being identified by the detectable labels of said longer oligodeoxynucleotides, said recognition sites being spaced apart by the determined length.

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127. The method of claim 123 wherein said determining step further comprises detecting the amplified DNA fragments by a method comprising labeling said fragments with a DNA intercalating dye or detecting light emission from a 25 fluorochrome label on said fragments.

128. The method of claim 123 further comprising, prior to said determining step, the step of hybridizing the amplified DNA fragments with a detectably labeled 30 oligodeoxynucleotide complementary to a subsequence, said subsequence differing from said recognition sites of said one or more restriction endonucleases, wherein the determining step further comprises detecting said detectable label of said oligodeoxynucleotide, and wherein a sequence from said 35 database matches a fragment of determined length when the sequence from said database further comprises said

subsequence between the recognition sites of said one or more restriction endonucleases.

129. The method of claim 123 wherein the one or more restriction endonucleases are pairs of restriction endonucleases, the pairs being selected from the group consisting of Acc56I and HindIII, Acc65I and NgoMI, BamHI and EcoRI, BglII and HindIII, BglII and NgoMI, BsiWI and BspHI, BspHI and BstYI, BspHI and NgoMI, BsrGI and EcoRI, EagI and 10 EcoRI, EagI and HindIII, EagI and NcoI, HindIII and NgoMI, NgoMI and NheI, NgoMI and SpeI, BglII and BspHI, Bsp120I and NcoI, BssHII and NgoMI, EcoRI and HindIII, and NgoMI and XbaI.

15 130. The method of claim 123 wherein the step of ligating is performed with T4 DNA ligase.

131. The method of claim 123 wherein the steps of digesting, contacting, and ligating are performed 20 simultaneously in the same reaction vessel.

132. The method of claim 123 wherein the steps of digesting, contacting, ligating, extending, and amplifying are performed in the same reaction vessel.

25 133. The method of claim 123 wherein the step of determining the length is performed by electrophoresis.

134. The method of claim 123 wherein the step of 30 searching said DNA database further comprises:

(a) determining a pattern of fragments that can be generated and for each fragment in said pattern those sequences in said DNA database that are capable of generating the fragment by simulating the steps of digesting with said 35 one or more restriction endonucleases, contacting, ligating, extending, amplifying, and determining applied to each sequence in said DNA database; and

(b) finding the sequences that are capable of generating said one or more fragments of determined length by finding in said pattern one or more fragments that have the same length and recognition sites as said one or more 5 fragments of determined length.

135. The method of claim 123 wherein the steps of digesting and ligating go substantially to completion.

10 136. The method of claim 123 wherein the DNA sample is cDNA of RNA from a tissue or a cell type derived from a plant, a single celled animal, a multicellular animal, a bacterium, a virus, a fungus, or a yeast.

15 137. The method of claim 123 wherein the DNA sample is cDNA of RNA from one or more cell types of a mammal.

138. The method of claim 137 wherein the mammal is a human.

20 139. The method of claim 137 wherein the mammal is a human having or suspected of having a diseased condition.

140. The method of claim 139 wherein the diseased 25 condition is a malignancy.

141. The method of claim 123 wherein said DNA sample is cDNA prepared from mRNA.

30 142. A method for identifying, classifying, or quantifying DNA molecules in a sample of DNA molecules with a plurality of nucleotide sequences, the method comprising the steps of:

(a) digesting said sample with one or more restriction 35 endonucleases, each said restriction endonuclease recognizing a subsequence recognition site and digesting DNA to produce fragments with 3' overhangs;

(b) contacting said fragments with shorter and longer oligodeoxynucleotides, each said longer oligodeoxynucleotide consisting of a first and second contiguous portion, said first portion being a 3' end subsequence complementary to the 5 overhang produced by one of said restriction endonucleases, each said shorter oligodeoxynucleotide complementary to the 3' end of said second portion of said longer oligodeoxynucleotide stand;

(c) ligating said longer oligodeoxynucleotides to said 10 DNA fragments to produce a ligated fragments;

(d) extending said ligated DNA fragments by synthesis with a DNA polymerase to form blunt-ended double stranded DNA fragments;

(e) amplifying said double stranded DNA fragments by 15 use of a DNA polymerase and primer oligodeoxynucleotides to produce amplified DNA fragments, each said primer oligodeoxynucleotide having a sequence comprising that of a longer oligodeoxynucleotide;

(f) determining the length of the amplified DNA 20 fragments; and

(g) searching a DNA sequence database, said database comprising a plurality of known DNA sequences that may be present in the sample, for sequences matching one or more of said fragments of determined length, a sequence from said 25 database matching a fragment of determined length when the sequence from said database comprises recognition sites of said one or more restriction endonucleases spaced apart by the determined length,

whereby DNA sequences in said sample are identified, 30 classified, or quantified.

143. A method of detecting one or more differentially expressed genes in an *in vitro* cell exposed to an exogenous factor relative to an *in vitro* cell not exposed to said 35 exogenous factor comprising:

(a) performing the method of claim 1 wherein said plurality of nucleic acids comprises cDNA of RNA of said in vitro cell exposed to said exogenous factor;

(b) performing the method of claim 1 wherein said 5 plurality of nucleic acids comprises cDNA of RNA of said in vitro cell not exposed to said exogenous factor; and

(c) comparing the identified, classified, or quantified cDNA of said in vitro cell exposed to said exogenous factor with the identified, classified, or quantified cDNA of said 10 in vitro cell not exposed to said exogenous factor, whereby differentially expressed genes are identified, classified, or quantified.

144. A method of detecting one or more differentially 15 expressed genes in a diseased tissue relative to a tissue not having said disease comprising:

(a) performing the method of claim 1 wherein said plurality of nucleic acids comprises cDNA of RNA of said diseased tissue, such that one or more cDNA molecules are 20 identified, classified, and/or quantified;

(b) performing the method of claim 1 wherein said plurality of nucleic acids comprises cDNA of RNA of said tissue not having said disease, such that one or more cDNA molecules are identified, classified, and/or quantified; and

25 (c) comparing said identified, classified, and/or quantified cDNA molecules of said diseased tissue with said identified, classified, and/or quantified cDNA molecules of said tissue not having the disease, whereby differentially expressed cDNA molecules are 30 detected.

145. The method of claim 144 wherein the step of comparing further comprises finding cDNA molecules which are reproducibly expressed in said diseased tissue or in said 35 tissue not having the disease and further finding which of said reproducibly expressed cDNA molecules have significant

differences in expression between the tissue having said disease and the tissue not having said disease.

146. The method of claim 145 wherein said finding cDNA molecules which are reproducibly expressed and said significant differences in expression of said cDNA molecules in said diseased tissue and in said tissue not having the disease are determined by a method comprising applying statistical measures.

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147. The method of claim 146 wherein said statistical measures comprise finding reproducible expression if the standard deviation of the level of quantified expression of a cDNA molecule in said diseased tissue or said tissue not having the disease is less than the average level of quantified expression of said cDNA molecule in said diseased tissue or said tissue not having the disease, respectively, and wherein a cDNA molecule has significant differences in expression if the sum of the standard deviation of the level of quantified expression of said cDNA molecule in said diseased tissue plus the standard deviation of the level of quantified expression of said cDNA molecule in said tissue not having the disease is less than the absolute value of the difference of the level of quantified expression of said cDNA molecule in said diseased tissue minus the level of quantified expression of said cDNA molecule in said tissue not having the disease.

148. The method of claim 144 wherein the diseased tissue and the tissue not having the disease are from one or more mammals.

149. The method of claim 144 wherein the disease is a malignancy.

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150. The method of claim 144 wherein the disease is a malignancy selected from the group consisting of prostrate

cancer, breast cancer, colon cancer, lung cancer, skin cancer, lymphoma, and leukemia.

151. The method of claim 144 wherein the disease is a 5 malignancy and the tissue not having the disease has a premalignant character.

152. A method of staging or grading a disease in a human individual comprising:

10 (a) performing the method of claim 1 in which said plurality of nucleic acids comprises cDNA of RNA prepared from a tissue from said human individual, said tissue having or suspected of having said disease, whereby one or more said cDNA molecules are identified, classified, and/or quantified; 15 and

(b) comparing said one or more identified, classified, and/or quantified cDNA molecules in said tissue to the one or more identified, classified, and/or quantified cDNA molecules expected at a particular stage or grade of said disease.

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153. A method for predicting a human patient's response to therapy for a disease, comprising:

(a) performing the method of claim 1 in which said plurality of nucleic acids comprises cDNA of RNA prepared 25 from a tissue from said human patient, said tissue having or suspected of having said disease, whereby one or more cDNA molecules in said sample are identified, classified, and/or quantified; and

(b) ascertaining if the one or more cDNA molecules 30 thereby identified, classified, and/or quantified correlates with a poor or a favorable response to one or more therapies.

154. The method of claim 153 which further comprises selecting one or more therapies for said patient for which 35 said identified, classified, and/or quantified cDNA molecules correlates with a favorable response.

155. A method for evaluating the efficacy of a therapy in a mammal having a disease, the method comprising:

(a) performing the method of claim 1 wherein said plurality of nucleic acids comprises cDNA of RNA of said 5 mammal prior to a therapy;

(b) performing the method of claim 1 wherein said plurality of nucleic acids comprises cDNA of RNA of said mammal subsequent to said therapy;

(c) comparing one or more identified, classified, 10 and/or quantified cDNA molecules of said mammal prior to said therapy with one or more identified, classified, and/or quantified cDNA molecules of said mammal subsequent to therapy; and

(d) determining whether the response to therapy is 15 favorable or unfavorable according to whether any differences in the one or more identified, classified, and/or quantified cDNA molecules after therapy are correlated with regression or progression, respectively, of the disease.

20 156. The method of claim 155 wherein the mammal is a human.

157. A kit comprising:

(a) one or more containers having one or more 25 restriction endonucleases;

(b) one or more containers having one or more shorter oligodeoxynucleotide strands;

(c) one or more containers having one or more longer oligodeoxynucleotide strands hybridizable with said shorter 30 strands, wherein either the longer or the shorter oligodeoxynucleotide strands each comprise a subsequence complementary to a single-stranded overhang produced by at least one of said one or more restriction endonucleases; and

(d) instructions packaged in association with said one 35 or more containers for use of said restriction endonucleases, shorter strands, and longer strands for identifying,

classifying, or quantifying one or more DNA molecules in a DNA sample, said instructions comprising:

- i. digest said sample with said restriction endonucleases into fragments, each fragment being terminated 5 on each end by a single-stranded overhang of said one or more restriction endonucleases;
- ii. contact said shorter and longer strands and said digested fragments to form double stranded DNA adapters annealed to said digested fragments,
- 10 iii. ligate said longer strand to said fragments;
- iv. generate one or more signals by separating and detecting such of said fragments that are digested on each end, each signal comprising a representation of the length of the fragment and the identity of the recognition sites on 15 both termini of the fragments; and
- v. search a nucleotide sequence database to determine sequences that match or the absence of any sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences 20 of nucleic acids that may be present in the sample, a sequence from said database matching a generated signal when the sequence from said database has both (i) the same length between occurrences of said recognition sites of said one or more restriction endonucleases as is represented by the 25 generated signal and (ii) the same recognition sites of said one of more restriction endonucleases as is represented by the generated signal.

158. The kit of claim 157 wherein said one or more 30 restriction endonucleases generate 5' overhangs at the terminus of digested fragments, wherein each said shorter oligodeoxynucleotide strand consists of a first and second contiguous portion, said first portion being a 5' end subsequence complementary to the overhang produced by one of 35 said restriction endonucleases, and wherein each said longer oligodeoxynucleotide strand comprises a 3' end subsequence

complementary to said second portion of said shorter oligodeoxynucleotide strand.

159. The kit of claim 157 wherein said one or more restriction endonucleases generate 3' overhangs at the terminus of the digested fragments, wherein each said longer oligodeoxynucleotide strand consists of a first and second contiguous portion, said first portion being a 3' end subsequence complementary to the overhang produced by one of 10 said restriction endonucleases, and wherein each said shorter oligodeoxynucleotide strand is complementary to the 3' end of said second portion of said longer oligodeoxynucleotide stand.

15 160. The kit of claim 157 wherein said instructions further comprise those signals expected from one or more DNA molecules of interest when said sample is digested with a particular one or more restriction endonucleases selected from among said one or more restriction endonucleases in said 20 kit.

161. The kit of claim 160 wherein said one or more DNA molecules of interest are cDNA molecules differentially expressed in a disease condition.

25 162. The kit of claim 157 wherein the restriction endonucleases are selected from the group consisting of Acc65I, AflIII, AgeI, ApaLI, ApoI, AscI, AvrI, BamHI, BclI, BglII, BsiWI, Bsp120I, BspEI, BspHI, BsrGI, BssHII, BstYI, 30 EagI, EcoRI, HindIII, MluI, NcoI, NgoMI, NheI, NotI, SpeI, and XbaI.

163. The kit of claim 157 which comprises one or more containers having one or more double stranded adapter DNA 35 molecules formed by annealing said longer and said shorter oligonucleotide strands.

164. The kit of claim 157 further comprising a computer readable memory according to claim 106.

165. The kit of claim 157 further comprising a computer readable memory according to claim 114.

166. The kit of claim 157 further comprising a computer readable memory according to claim 122.

10 167. The kit of claim 157 further comprising in a container a DNA ligase.

168. The kit of claim 157 further comprising in a container a phosphatase capable of removing terminal 15 phosphates from a DNA sequence.

169. The kit of claim 157 further comprising in one or more containers:

(a) one or more primers, each said primer consisting of 20 a single stranded oligodeoxynucleotide comprising the sequence of one of said longer strands; and
(b) a DNA polymerase.

170. The kit of claim 169 wherein each of said one or 25 more primers further comprises (a) a first subsequence that is the portion of the recognition site of one of said one or more restriction endonucleases remaining at the terminus of a fragment after digestion, and (b) a second subsequence of one or two additional nucleotides contiguous with and 3' to said 30 first subsequence, wherein said primer is detectably labeled such that primers with differing said one or two additional nucleotides have different labels that can be distinguishably detected.

35 171. The kit of claim 157 wherein said instructions further comprise: detect such of said fragments digested on each end by a method comprising staining said fragments with

silver, labeling said fragments with a DNA intercalating dye, or detecting light emission from a fluorochrome label on said fragments.

5 172. The kit of claim 157 further comprising:

- (a) reagents for performing a cDNA sample preparation step;
- (b) reagents for performing a step of digestion by one or more restriction endonucleases;
- 10 (c) reagents for performing a ligation step; and
- (d) reagents for performing a PCR amplification step.

173. A method for identifying, classifying, or quantifying one or more nucleic acids in a sample comprising 15 a plurality of nucleic acids having different nucleotide sequences, said method comprising:

- (a) probing said sample with one or more recognition means, each recognition means causing recognition of a target nucleotide subsequence or a set of target nucleotide 20 subsequences;
- (b) generating one or more signals from said sample probed by said recognition means, each generated signal arising from a nucleic acid in said sample and comprising a representation of (i) the identities of effective 25 subsequences, each said effective subsequence being a subsequence comprising a target subsequence, or the identities of sets of effective subsequences, each said set having member effective subsequences each of which comprises a different target subsequence from one of said sets of 30 target sequences, and (ii) the length between occurrences of effective subsequences in said nucleic acid or between one occurrence of one effective subsequence and the end of said nucleic acid; and
- (c) searching a nucleotide sequence database to 35 determine sequences that match or the absence of any sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences

of nucleic acids that may be present in the sample, a sequence from said database matching a generated signal when the sequence from said database has both (i) the same length between occurrences of effective subsequences or the same 5 length between one occurrence of one effective target subsequence and the end of the sequence as is represented by the generated signal, and (ii) the same effective subsequences as are represented by the generated signal, or effective subsequences that are members of the same sets of 10 effective subsequences as are represented by the generated signal,

whereby said one or more nucleic acids in said sample are identified, classified, or quantified.

15 174. The method according to claim 173 wherein said one or more nucleic acids are DNA.

175. The method according to claim 174 wherein one or more of said effective subsequences consist of a single 20 target nucleotide subsequence.

176. The method according to claim 175 wherein each of said single target nucleotide subsequences is recognized by a first restriction endonuclease having said single target 25 nucleotide subsequence for its recognition site.

177. The method according to claim 174 wherein one or more of said effective subsequences consist of two target nucleotide subsequences, a first target nucleotide 30 subsequence and a second target nucleotide subsequence.

178. The method according to claim 177 wherein each of said first target nucleotide subsequences is recognized by a restriction endonuclease having said first target nucleotide 35 subsequence for its recognition site.

179. The method according to claim 177 wherein each of said second target nucleotide subsequences is at least a portion of a single-stranded overhang produced by a Type IIS restriction endonuclease.

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180. The method according to claim 177 wherein said first target nucleotide subsequence and said second target nucleotide subsequence are adjacent in said one or more said effective subsequences.

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181. The method according to claim 174 further comprising:

(a) identifying a fragment of a nucleic acid in said sample which generates one or more of said signals; and

15 (b) recovering said fragment.

182. The method according to claim 181 which further comprises using at least a hybridizable portion of said fragment as a hybridization probe to hybridize to a nucleic acid that comprises said fragment.

183. The method according to claim 174 wherein one or more of said signals do not match any sequence in said nucleotide sequence database.

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184. The method according to claim 174 wherein said DNA is cDNA synthesized from source mRNA according to a synthesis method comprising using one or more primers with a conjugated capture moiety.

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185. The method according to claim 174 wherein said DNA is cDNA synthesized from source mRNA, and wherein said end of said nucleic acid has a fixed offset from a 5'-cap of said source mRNA.

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186. The method according to claim 185 wherein said synthesis is according to a method comprising a step of

ligating a DNA-RNA chimera to said source mRNA in a fixed offset to said 5'-cap of said source mRNA.

187. The method according to claim 186 wherein said 5 ligating in a fixed offset comprises ligating to the ribonucleotide 3' adjacent to the 5' cap ribonucleotide.

188. The method according to claim 186 wherein said DNA-RNA chimera comprises a DNA portion having a conjugated 10 capture moiety.

189. The method according to claim 188 wherein said probing step further comprises a step of digesting said sample into fragments and subsequent steps of contacting said 15 fragments with a binding partner of said capture moiety affixed to a solid support, washing said binding partner, and denaturing DNA bound to said binding partner to release strands not having a conjugated capture moiety, and wherein said generating step generates signals from said released 20 strands.

190. The method according to claim 174 wherein said DNA is cDNA synthesized from source mRNA, and wherein said end of said nucleic acid has a fixed offset from the 5' end of the 25 3' poly(A) tail of said source mRNA.

191. The method according to claim 190 wherein said synthesis is according to a method comprising a step of synthesizing a cDNA strand with a nucleotide polymerase 30 primed by oligo(dT) phasing primers.

192. The method according to claim 174 wherein one or more of said recognition means are Type IIS restriction endonucleases, wherein the step of probing further comprises 35 a first digesting step in which said sample is digested with said one or more Type IIS restriction endonucleases, thereby creating first single-stranded overhangs on cut fragments

outside of the recognition sites of the Type IIS restriction endonucleases, and wherein one or more of said effective subsequences comprise a first target nucleotide subsequence that is the sequence of at least a portion of one of said 5 first single-stranded overhangs.

193. The method according to claim 174 wherein one or more of said recognition means are restriction endonucleases, wherein the step of probing further comprises a first 10 digesting step in which said sample is digested with said one or more restriction endonucleases, thereby creating first single-stranded overhangs on cut fragments, and wherein one or more of said effective subsequences comprise a first target nucleotide subsequence that is a recognition site of 15 one of said restriction endonucleases.

194. The method according to claim 193 wherein said step of probing further comprises after said digesting step: a step of hybridizing partially double-stranded adapter 20 nucleic acids with said fragments, each said adapter nucleic acid comprising a primer strand and a linker strand, each said linker oligonucleotide (i) being shorter in sequence than said primer oligonucleotide, and (ii) having an end complementary to one of said first single-stranded overhangs; 25 and

a step of ligating said primer strand of said hybridized adapter nucleic acid to said first single-stranded overhang, whereby ligated fragments are formed.

195. The method according to claim 194 wherein said step of probing further comprises after said steps of hybridizing and ligating a step of amplifying said ligated fragments, whereby amplified fragments are formed.

196. The method according to claim 195 wherein said amplifying is carried out by use of a nucleic acid polymerase and primers that are single-stranded nucleic acids comprising

the sequence of said primer strands of said adapter nucleic acids, said primers being capable of priming nucleic acid synthesis by said polymerase.

5 197. The method according to claim 196 wherein said primers further comprise a conjugated capture moiety and a release means, and wherein said step of probing further comprises after said step of amplifying a step of cleanup, which comprises contacting said amplified fragments with a 10 binding partner of said capture moiety affixed to a solid support, washing said binding partner in denaturing conditions, and using said release means to release fragments bound to said binding partner, whereby released fragments are formed, and wherein said generating step generates signals 15 from said released fragments.

198. The method according to claim 196 wherein said primers further comprise a conjugated capture moiety, and wherein said step of probing further comprises after said 20 step of amplifying a step of cleanup, which comprises contacting said amplified fragments with a binding partner of said capture moiety affixed to a solid support, washing said binding partner, and denaturing DNA bound to said binding partner to release fragments not having a conjugated capture 25 moiety, whereby released fragments are formed, and wherein said generating step generates signals from said released fragments.

199. The method according to claim 198 wherein said 30 capture moiety is biotin.

200. The method according to claim 198 wherein said step of generating further comprises separating said released fragments according to length and detecting the lengths of 35 said fragments.

201. The method according to claim 200 wherein said separating is done by electrophoresis in denaturing conditions.

5 202. The method according to claim 194 wherein said step of generating further comprises a step of separating said ligated fragments according to length and detecting the lengths of such fragments.

10 203. The method according to claim 202 wherein said primer strands of said adapter nucleic acids are distinguishably labeled, and wherein said step of generating further comprises detecting said labels of said primer strands.

15 204. The method according to claim 202 wherein prior to said generating step said ligated fragments are amplified.

20 205. The method according to claim 194 wherein one or more of said recognition means are Type IIS restriction endonucleases, wherein the step of probing further comprises a second digesting step in which said sample is digested with said one or more Type IIS restriction endonucleases, thereby creating second single-stranded overhangs on cut fragments 25 outside of the recognition sites of the Type IIS restriction endonucleases, and wherein one or more of said effective subsequences comprise a second target nucleotide subsequence that is the sequence of at least a portion of one of said second single-stranded overhangs.

30 206. The method according to claim 205 wherein said primer strands of said adapter nucleic acids comprise a recognition site for a Type IIS restriction endonuclease.

35 207. The method according to claim 205 wherein said primer strands of said adapter nucleic acids comprise a recognition site for a Type IIS restriction endonuclease so

positioned that said first target nucleotide subsequence and said second target nucleotide subsequence are adjacent in said one or more said effective subsequences.

5 208. The method according to claim 205 wherein the step of probing further comprises before said second digesting step a step of amplifying said ligated fragments.

10 209. The method according to claim 205 wherein said primer strands further comprise a conjugated capture moiety, and wherein the step of probing further comprises before said second digestion step a step which comprises contacting said ligated fragments with a binding partner of said capture moiety affixed to a solid support, washing said binding 15 partner, and denaturing DNA bound to said binding partner to release fragments not having a conjugated capture moiety, whereby released fragments are formed, and wherein said generating step generates signals from said released fragments.

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210. The method according to claim 205 wherein said generating step further comprises a step of determining the sequences of said second single-stranded overhangs.

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211. The method according to claim 210 wherein said step of determining the sequence comprises performing Sanger sequencing reactions on the second single-stranded overhangs primed by the shorter strand of said fragments with said second single-stranded overhangs.

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212. The method according to claim 211 wherein said primer strand ligated to said shorter strand of said fragments with said second single-stranded overhangs comprises a release means, and wherein said step of 35 determining the sequence further comprises after said performing Sanger sequencing reactions release of said shorter strand by said release means.

213. The method according to claim 212 wherein said release means comprises a subsequence of one or more uracil nucleotides or a recognition site for a rare cutting restriction endonuclease.

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214. The method according to claim 213 wherein said rare cutting restriction endonuclease is Ascl.

215. The method according to claim 210 wherein said 10 step of determining the sequence comprises performing a method comprising:

(a) hybridizing partially double-stranded second adapter nucleic acids to said second single-stranded overhangs, each said second adapter nucleic acid comprising a 15 second primer oligonucleotide strand and a second phasing linker oligonucleotide strand, each said second phasing linker oligonucleotide strand being shorter in sequence than said second primer oligonucleotide strand, and having one fixed nucleotide hybridizable with one position of said 20 second single-stranded overhangs and random nucleotides hybridizable with remaining positions of said second single-stranded overhangs, said second primer oligonucleotide strand being distinctively labeled according to the identity of said one fixed nucleotide;

25 (b) ligating said second primer oligonucleotide strands to said overhangs with a ligase;

(c) detecting which said second primer oligonucleotide strand has been ligated to said overhangs and thereby determining which fixed nucleotide hybridizes with 30 said one position of said second single-stranded overhang; and

(d) repeating steps (a) through (c) for all possible nucleotides at all possible positions of said second single-stranded overhang;

35 whereby the nucleotide sequence of said second single-stranded overhang is determined.

216. A method for identifying, classifying, or quantifying DNA molecules in a sample comprising DNA molecules having a plurality of different nucleotide sequences, the method comprising the steps of:

5 (a) digesting said sample with one or more first restriction endonucleases, each said first restriction endonuclease cutting DNA molecules in said sample at its recognition site to produce fragments with first single-stranded overhangs of known sequence;

10 (b) hybridizing said fragments with linker oligonucleotides and first primer oligonucleotides, each said linker oligonucleotide hybridizable with one of said first single-stranded overhangs, and each said first primer oligonucleotide hybridizable with one of said linker oligonucleotides;

15 (c) ligating said first primer oligonucleotides to the end of said first single-stranded overhangs, whereby ligated fragments are formed;

(d) amplifying said ligated fragments to produce

20 amplified fragments by a method comprising contacting said ligated fragments with a DNA polymerase and second primer oligonucleotides, each said second primer oligonucleotide having a sequence comprising that of one of said first primer oligonucleotides, and wherein one of said second primer oligonucleotides has a conjugated capture moiety, and one of said second primer oligonucleotides comprises a recognition site for a Type IIS restriction endonuclease, whereby

25 amplified fragments are formed;

(e) binding said amplified fragments by contacting said amplified fragments with the binding partner of said capture moiety affixed to a solid support, whereby bound fragments are formed;

30 (f) washing at least a portion of said bound fragments;

(g) denaturing at least a portion of said bound washed fragments to release strands not conjugated to said capture moiety;

(h) determining the length of at least one of said released strands, thereby determining one or more fragments of determined length;

(i) digesting at least a portion of said bound, washed 5 fragments with said Type IIS restriction endonuclease to produce fragments with second single-stranded overhangs; and

(j) sequencing said second single-stranded overhangs created in step (i).

10 217. The method according to claim 216 wherein said recognition site for said Type IIS restriction endonuclease is so positioned that upon cleavage with said Type IIS restriction endonuclease one of said second single-stranded overhangs is created adjacent to the recognition site of said 15 first restriction endonuclease, and said method further comprising, after said sequencing step, a step of searching a DNA sequence database for sequences matching or the absence of any sequences matching one or more of said fragments of determined length, said database comprising a plurality of 20 known DNA sequences that may be present in the sample, a sequence from said database matching a fragment of determined length when the sequence from said database comprises effective subsequences spaced apart by said determined length, said effective subsequences consisting of said 25 recognition sites of said one or more first restriction endonucleases concatenated to sequences of said second single-stranded overhangs.

218. The method according to claim 216 wherein said 30 recognition site for said Type IIS restriction endonuclease is so positioned that upon cleavage with said Type IIS restriction endonuclease one of said second single-stranded overhangs is created non-contiguous with the recognition site of said first restriction endonuclease, and said method 35 further comprising, after said sequencing step, a step of searching a DNA sequence database for sequences matching or the absence of any sequences matching one or more of said

fragments of determined length, said database comprising a plurality of known DNA sequences that may be present in the sample, a sequence from said database matching a fragment of determined length when the sequence from said database 5 comprises the same recognition sites of said one or more first restriction endonucleases spaced apart by said determined length.

219. The method according to claim 218 wherein a 10 sequence from said database matches a fragment of determined length only when said sequence from said database further comprises the sequence of said second single-stranded overhang spaced apart from said recognition site in said sequence by the same number of nucleotides as the second 15 single-stranded overhang is spaced apart from said recognition site in said fragment of determined length.

220. A method for identifying, classifying, or quantifying cDNA molecules in a sample comprising cDNA 20 molecules having a plurality of different nucleotide sequences, the method comprising the steps of:

(a) digesting said sample with one or more first restriction endonucleases, each said first restriction endonuclease cutting cDNA molecules in said sample at its 25 recognition site to produce fragments with single-stranded overhangs of known sequence;

(b) hybridizing said fragments with linker oligonucleotides and first primer oligonucleotides, each said linker oligonucleotide hybridizable with one of said single- 30 stranded overhangs, and each said first primer oligonucleotide hybridizable with one of said linker oligonucleotides;

(c) ligating said first primer oligonucleotides to the end of said single-stranded overhangs, whereby ligated 35 fragments are formed;

(d) amplifying said ligated fragments to produce amplified fragments by a method comprising contacting said

ligated fragments with a DNA polymerase and second primer oligonucleotides, one of said second primer oligonucleotides having a conjugated capture moiety and a sequence comprising that of a 5'-cap-primer oligonucleotide, and each of the 5 remaining of said second primer oligonucleotides having a sequence comprising that of one of said first primer oligonucleotides, wherein said sample of cDNA was synthesized from source mRNA by a method such that cDNA molecules in said sample comprise said 5'-cap-primer oligonucleotide in a fixed 10 relation to the 5'-cap of said source mRNA; whereby amplified fragments are formed;

(e) binding said amplified fragments by contacting said amplified fragments with the binding partner of said capture moiety affixed to a solid support, whereby bound fragments 15 are formed;

(f) washing at least a portion of said bound fragments;

(g) denaturing at least a portion of said bound, washed fragments to release strands not conjugated to said capture moiety; and

20 (h) determining the length of at least one of said released strands, thereby determining one or more fragments of determined length.

221. A method for identifying, classifying, or 25 quantifying DNA molecules in a sample comprising DNA molecules having a plurality of different nucleotide sequences, the method comprising the steps of:

(a) digesting said sample with one or more first restriction endonucleases, each said first restriction 30 endonuclease cutting DNA molecules in said sample at its recognition site to produce fragments with single-stranded overhangs of known sequence;

(b) hybridizing said fragments with linker oligonucleotides and first primer oligonucleotides, each said 35 linker oligonucleotide hybridizable with one of said single-stranded overhangs, and each said first primer

oligonucleotide hybridizable with one of said linker oligonucleotides;

(c) ligating said first primer oligonucleotides to the end of said single-stranded overhangs; whereby ligated 5 fragments are formed;

(d) amplifying said ligated fragments to produce amplified fragments by a method comprising contacting said ligated fragments with a DNA polymerase and second primer oligonucleotides, each said second primer oligonucleotide 10 having a sequence comprising that of one of said first primer oligonucleotides, and wherein one of said second primer oligonucleotides comprises a conjugated capture moiety; whereby amplified fragments are formed;

(e) binding said amplified fragments by contacting said 15 amplified fragments with the binding partner of said capture moiety affixed to a solid support; whereby bound fragments are formed;

(f) washing at least a portion of said bound fragments;

(g) denaturing at least a portion of said bound, washed 20 fragments to release strands not conjugated to said capture moiety; and

(h) determining the length of at least one of said released strands, thereby determining one or more fragments of determined length.

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222. The method according to claim 221 wherein said linker oligonucleotide is shorter in sequence than said first primer oligonucleotide.

30 223. The method according to claim 221 further comprising prior to said hybridizing step a step of hybridizing said linker oligonucleotides and said first primer oligonucleotides to form partially double stranded adapter nucleic acids, and wherein said hybridizing step 35 comprises hybridizing said adapter nucleic acids to said overhangs.

224. The method according to claim 221 further comprising prior to said amplifying step a step of extending said ligated DNA fragments to blunt-ended double stranded DNA fragments by synthesis with a DNA polymerase.

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225. The method according to claim 221 wherein the steps of digesting, hybridizing, and ligating are performed simultaneously in a first reaction mix, and wherein the step of amplifying is performed in a second reaction mix.

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226. The method according to claim 225 wherein said first and said second reaction mixes are in the same reaction vessel separated by a separation means during said steps of digesting, hybridizing, ligating, and amplifying, and wherein 15 the step of amplifying further comprises a step of causing said first and said second reaction mixes to combine across said separation means.

227. The method according to claim 226 wherein said 20 separation means is a wax, and wherein said amplifying step melts said wax.

228. The method according to claim 227 wherein said wax is a mixture of Paraffin and Chillout™ waxes.

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229. The method according to claim 221 wherein said determining step further comprises separating said released strands according to length by electrophoresis.

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230. The method according to claim 221 wherein said determining step further comprises detecting said release strands by a method comprising labeling said fragments with a DNA intercalating dye or Ag staining or detecting light emission from a fluorochrome label on said fragments.

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231. The method according to claim 221 wherein said one or more first restriction endonucleases are pairs of

restriction endonucleases, the pairs being selected from the group consisting of Acc56I and HindIII, Acc65I and NgoMI, BamHI and EcoRI, BglII and HindIII, BglII and NgoMI, BsiWI and BspHI, BspHI and BstYI, BspHI and NgoMI, BsrGI and EcoRI, 5 EagI and EcoRI, EagI and HindIII, EagI and NcoI, HindIII and NgoMI, NgoMI and NheI, NgoMI and SpeI, BglII and BspHI, Bsp120I and NcoI, BssHII and NgoMI, EcoRI and HindIII, and NgoMI and XbaI.

10 232. The method according to claim 221 wherein the step of ligating is performed with T4 DNA ligase.

233. The method according to claim 221 wherein said capture moiety is biotin.

15 234. The method according to claim 221 further comprising after said determining step a step of searching a DNA sequence database for sequences matching or the absence of any sequences matching one or more of said fragments of 20 determined length, said database comprising a plurality of known DNA sequences that may be present in the sample, a sequence from said database matching a fragment of determined length when the sequence from said database comprises the same recognition sites of said one or more restriction 25 endonucleases present in the fragment and spaced apart by the same determined length.

235. The method according to claim 234 wherein the step of searching said DNA database further comprises:

30 (a) determining a pattern of simulated fragments that can be generated from sequences in said DNA database and for each simulated fragment in said pattern those sequences in said DNA database that are capable of generating the fragment by simulating the steps of digesting with said one or more 35 restriction endonucleases, hybridizing, ligating, amplifying, and determining applied to each sequence in said DNA database; and

(b) finding the sequences in said database that are capable of generating said one or more fragments of determined length by finding in said pattern one or more fragments that have the same recognition sites spaced apart 5 by the same length as said one or more fragments of determined length.

236. The method according to claim 221 wherein the steps of digesting and ligating go substantially to 10 completion.

237. The method according to claim 221 wherein the DNA sample is cDNA of RNA from a tissue or a cell type derived from a plant, a single celled animal, a multicellular animal, 15 a bacterium, a virus, a fungus, or a yeast.

238. A method of detecting the presence of differentially expressed cDNAs in a first tissue relative to a second tissue comprising:

20 (a) performing the method of claim 218 wherein said sample of DNA molecules comprises cDNA of RNA of said first tissue, and wherein lengths are determined for one or more released strands from said first tissue;

(b) performing the method of claim 218 wherein said 25 sample of DNA molecules comprises cDNA of RNA of said second tissue, and wherein lengths are determined for one or more released strands from said second tissue; and

(c) comparing said released strands of determined 30 length from said first tissue with said released strands of determined length from said second tissue.

whereby the presence of differentially expressed cDNA molecules are detected.

239. The method according to claim 238 wherein the step 35 of comparing further comprises finding which of said released strands which are reproducibly expressed in said first tissue or in said second tissue and further finding which of said

reproducibly expressed released strands have significant differences in expression between said first tissue and said second tissue.

5 240. The method according to claim 239 further comprising:

- (a) identifying said released strands having significant differences in expression; and
- (b) recovering said differently expressed fragment.

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241. The method according to claim 240 which further comprises using at least a hybridizable portion of said released strands having significant differences in expression as a hybridization probe to bind to a nucleic acid that can 15 generate said released strands having significant differences in expression according to the method of claim 218.

242. The method according to claim 239 wherein said finding released strands which are reproducibly expressed and 20 said finding significant differences in expression of said released strands in said first tissue and in said second tissue are determined by a method comprising applying statistical measures.

25 243. The method according to claim 242 wherein said statistical measures comprise finding reproducible expression if the standard deviation of the level of quantified expression of a released strand in said first tissue or said second tissue is less than the average level of quantified 30 expression of said released strand in said first tissue or said second tissue, respectively, and wherein a released strand has significant differences in expression if the sum of the standard deviation of the level of quantified expression of said released strand in said first tissue plus 35 the standard deviations of the level of quantified expression of said released strand in said second tissue is less than the absolute value of the difference of the level of

quantified expression of said released strand in said first tissue minus the level of quantified expression of said released strand in said second tissue.

5 244. The method according to claim 238 wherein said first tissue and said second tissue are different tissue-types from the same organism.

10 245. The method according to claim 238 wherein said first tissue and said second tissue are the same tissue-type from phylogenetically related organisms.

15 246. The method according to claim 238 wherein said first tissue and said second tissue are the same tissue-type from an organism in a first condition and said organism in a second condition.

20 247. The method according to claim 246 wherein said first condition is a normal condition and said second condition is a diseased condition.

248. A kit comprising:

(a) one or more containers having one or more restriction endonucleases;

25 (b) one or more containers having one or more shorter oligodeoxynucleotide strands;

(c) one or more containers having one or more longer oligodeoxynucleotide strands hybridizable with said shorter strands, wherein either the longer or the shorter

30 oligodeoxynucleotide strands each comprise a sequence complementary to an overhang produced by at least one of said one or more restriction endonucleases, and wherein one or more of said longer oligodeoxynucleotide strands have a conjugated capture moiety; and

35 (d) instructions packaged in association with said one or more containers for use of said restriction endonucleases, shorter strands, and longer strands for identifying,

classifying, or quantifying one or more DNA molecules in a DNA sample, said instructions comprising:

i. digest said sample with said restriction endonucleases into fragments, each fragment being terminated 5 on each end by a recognition site of said one or more restriction endonucleases;

ii. contact said shorter and longer strands and said digested fragments to form double stranded DNA adapters annealed to said digested fragments,

10 iii. ligate said longer strand to said digested fragments such that each digested fragment has ligated to it one of said longer strands with a conjugated capture moiety;

iv. contact said digested fragments to a binding partner of said capture moiety, wash, and denature single 15 strands not conjugated to a capture moiety from said binding partner;

v. generate one or more signals from said 20 denatured single strands, each signal comprising a representation of the length of the fragment and the identity of the recognition sites on both termini of the fragments;

vi. search a nucleotide sequence database to determine sequences that match or the absence of any sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences 25 of nucleic acids that may be present in the sample, a sequence from said database matching a generated signal when the sequence from said database has both (1) the same length between occurrences of said recognition sites of said one or more restriction endonucleases as is represented by the 30 generated signal, and (2) the same recognition sites of said one or more restriction endonucleases as is represented by the generated signal.

249. The kit of claim 248 which comprises one or more 35 containers having one or more double stranded adapter DNA molecules formed by annealing said longer and said shorter oligonucleotide strands.

250. A kit comprising:

- (a) one or more containers having one or more non-Type IIS restriction endonucleases;
- (b) one of more containers having one or more Type IIS 5 restriction endonucleases;
- (c) one or more containers having one or more shorter oligodeoxynucleotide strands;
- (d) one or more containers having one or more longer oligodeoxynucleotide strands hybridizable with said shorter 10 strands, wherein either the longer or the shorter oligodeoxynucleotide strands each comprise a sequence complementary to an overhang produced by at least one of said one or more non-Type IIS restriction endonucleases, and wherein one or more of said longer oligodeoxynucleotide 15 strands has a recognition site for one of said Type IIS restriction endonucleases; and
- (e) instructions packaged in association with said one or more containers for use of said non-Type IIS restriction endonucleases, Type IIS restriction endonucleases, shorter 20 strands, and longer strands for identifying, classifying, or quantifying one or more DNA molecules in a DNA sample, said instructions comprising:
 - i. digest said sample with said non-Type IIS restriction endonucleases into fragments, each fragment being 25 terminated on each end by a recognition site of said one or more non-Type IIS restriction endonucleases;
 - ii. contact said shorter and longer strands and said digested fragments to form double stranded DNA adapters annealed to said digested fragments,
 - 30 iii. ligate said longer strand to said fragments such that each fragment has one said longer strand with one of said Type IIS recognition sites;
 - iv. separate such of said fragments that are digested on each end, and detect a representation of the 35 length of the fragments and the identity of the recognition sites of said non-Type IIS restriction endonucleases on both termini of the fragments;

v. digest said fragments with one of said Type IIS restriction endonucleases to produce second single-stranded overhangs;

5 vi. determine sequences of said second single-stranded overhangs produced on said fragments by said Type IIS restriction endonucleases;

vii. generate one or more signals, each signal comprising said representation of the length of the fragment and the identity of effective subsequences on both termini of 10 the fragments, said effective subsequence consisting either (1) of the sequence of said non-Type IIS restriction endonuclease recognition site for the terminus not further digested by said Type IIS restriction endonuclease, or (2) of said non-Type IIS restriction endonuclease recognition site 15 combined with said sequence of said second single-stranded overhang for the terminus further digested by said Type IIS restriction endonuclease; and

viii. search a nucleotide sequence database to determine sequences that match or the absence of any 20 sequences that match said one or more generated signals, said database comprising a plurality of known nucleotide sequences of nucleic acids that may be present in the sample, a sequence from said database matching a generated signal when the sequence from said database has both (1) the same length 25 between occurrences of said recognition sites of said one or more non-Type IIS restriction endonucleases as is represented by the generated signal and (2) the same effective subsequences as is represented by the generated signal.

30 251. The kit of claim 250 which comprises one or more containers having one or more double stranded adapter DNA molecules formed by annealing said longer and said shorter oligonucleotide strands.

35 252. The kit of claim 250 wherein said recognition sites of said Type IIS restriction endonucleases are so positioned on said longer oligodeoxynucleotide strands so

that said second single-stranded overhangs are positioned adjacent to said recognition site of said first restriction endonucleases.

5 253. A partially double stranded oligodeoxynucleotide comprising a first and a second oligodeoxynucleotide strand, said first strand oligodeoxynucleotide comprising an end complementary to a first single-stranded overhang produced by cleavage of a DNA molecule by a restriction endonuclease that 10 cuts said DNA molecule within a recognition site of said restriction endonuclease, and said partially double stranded oligodeoxynucleotide comprising a binding site for a Type IIS restriction endonuclease, said binding site so positioned with respect to 15 said end such that, when said partially double-stranded oligodeoxynucleotide is ligated to said DNA molecule cleaved by said restriction endonuclease, further cleavage by said Type IIS restriction endonuclease produces a second single-stranded overhang of said DNA molecule that is contiguous 20 with the recognition site of said restriction endonuclease.

254. The partially double stranded oligodeoxynucleotide of claim 253 having a melting temperature no greater than 68 °C.

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255. The partially double stranded oligodeoxynucleotide of claim 253 wherein the melting temperature of the second oligodeoxynucleotide strand from a strand complementary to said second oligodeoxynucleotide strand is greater than the 30 melting temperature of said first oligodeoxynucleotide strand from a strand complementary to said first oligodeoxynucleotide strand.

256. A partially double stranded oligodeoxynucleotide 35 comprising:

(a) a first oligodeoxynucleotide strand comprising a binding means and a release means; and

(b) a second oligodeoxynucleotide strand, said second oligodeoxynucleotide strand comprising

(i) a first subsequence complementary to a portion of said first oligodeoxynucleotide strand, and

5 (ii) a second subsequence at the 5' end complementary to a first single-stranded overhang produced by cleavage of a DNA molecule by a restriction endonuclease that cuts said DNA molecule within a recognition site of the restriction endonuclease.

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257. The partially double stranded oligodeoxynucleotide of claim 256 wherein said release means comprises a subsequence of said first oligodeoxynucleotide strand that is a recognition site for a restriction endonuclease that cuts 15 rarely in the mammalian genome.

258. The partially double stranded oligodeoxynucleotide of claim 256 wherein said release means comprises a subsequence of one or more uracil nucleotides.

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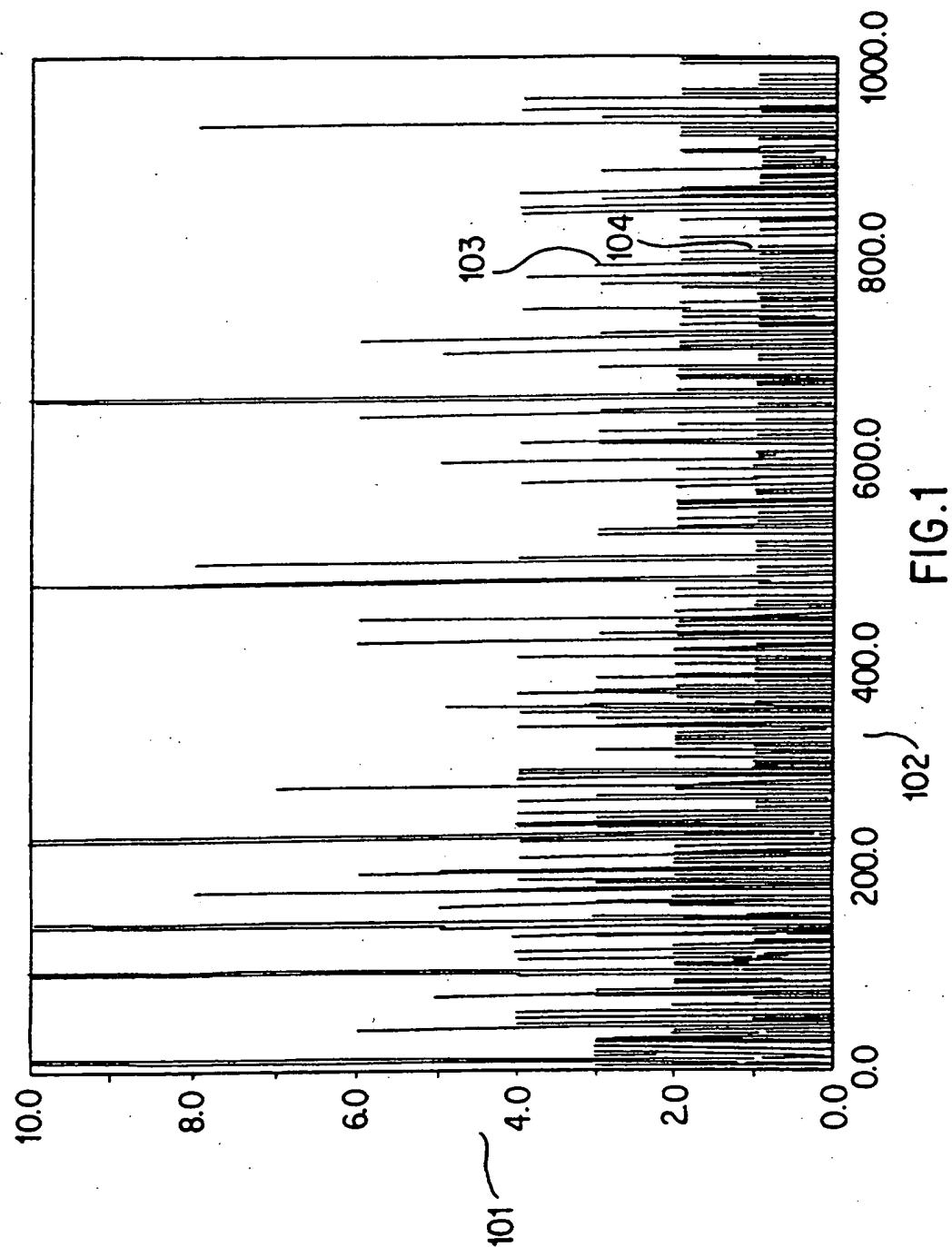
259. The partially double stranded oligodeoxynucleotide of claim 256 wherein said binding means comprises a biotin moiety attached to said first oligodeoxynucleotide strand.

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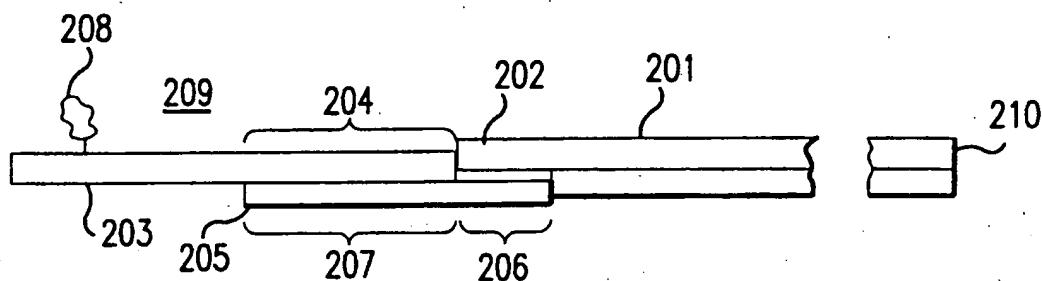


FIG.2A

5'-AGC ACT CTC CAG CCT CTC ACC GAA-3' (SEQ ID NO:1)

250 3'- AG TGG CTT CTAG-5' (SEQ ID NO:7)

5'-ACC GAC GTC GAC TAT CCA TGA AGC-3' (SEQ ID NO:42)

251 3'-GT ACT TCG TCGA-5' (SEQ ID NO:44)

FIG.2B

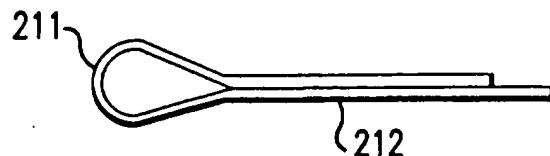


FIG.2C

SUBSTITUTE SHEET (RULE 26)

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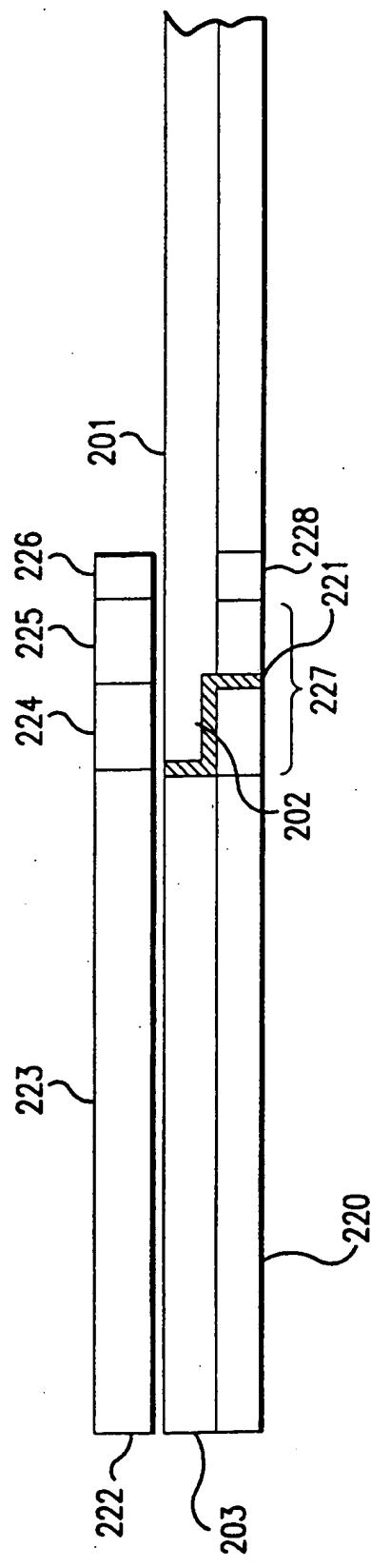


FIG. 2D

SUBSTITUTE SHEET (RULE 26)

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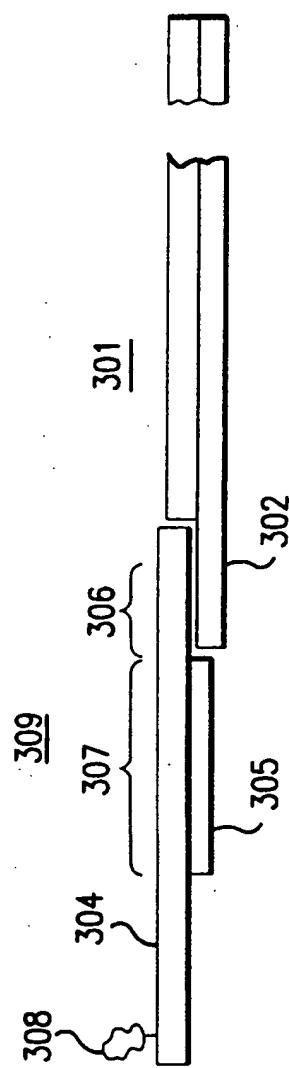


FIG. 3A

5' -AGCACTCTCCAGCCCTCACCGACCATG (SEQ ID NO.55)
3' -ACTGGCTC

FIG. 3B

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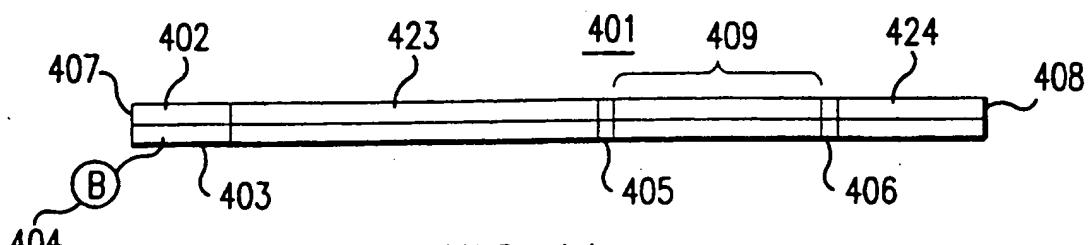


FIG. 4A

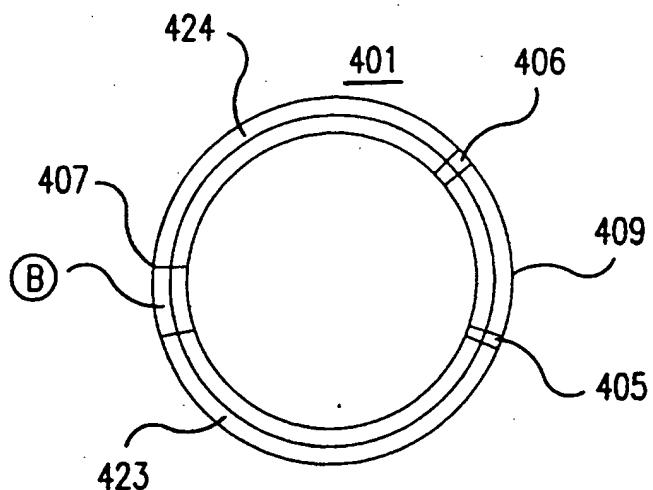


FIG. 4B

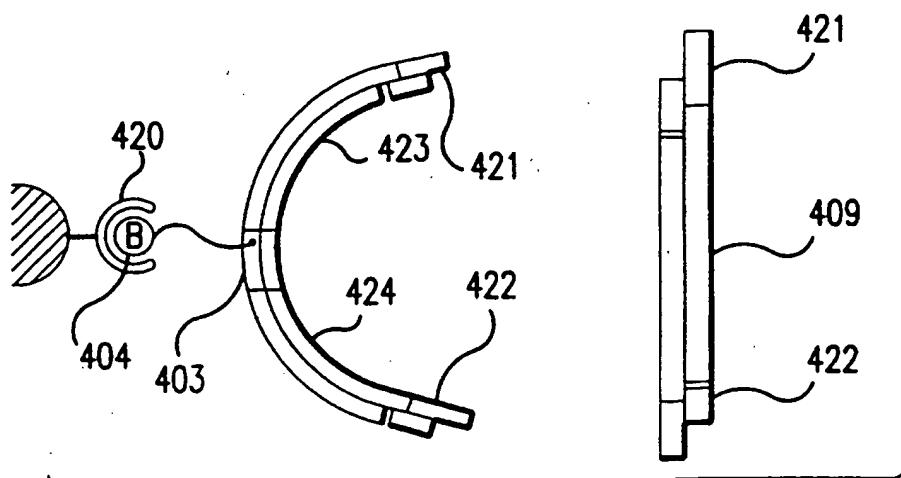


FIG. 4C

SUBSTITUTE SHEET (RULE 26)

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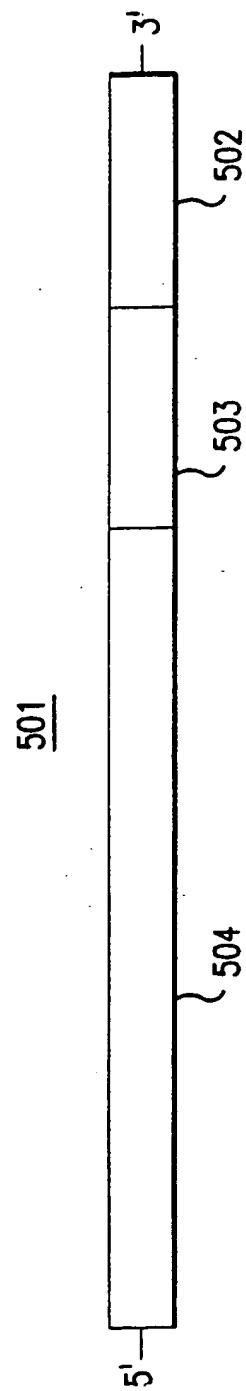


FIG. 5

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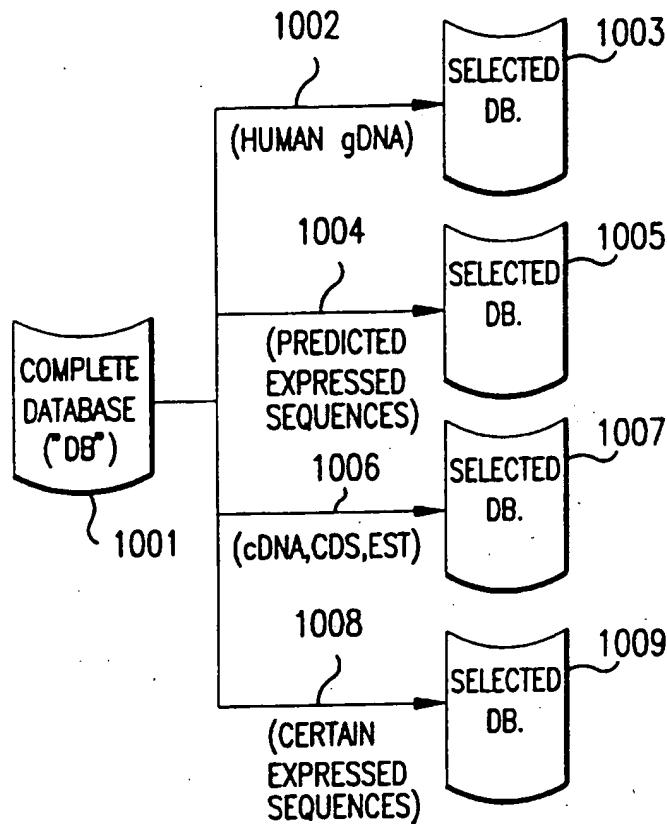


FIG. 6A

1011	1012	1013				
1014	A01	HUMAN	...	TAUT
,	,	,	,	,	,	,
,	,	,	,	,	,	,
,	,	,	,	,	,	,

1010

FIG. 6B

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1101	TISSUE SAMPLE		
1102	REACTION 1	RE1	LABEL1
		RE2	LABEL2
1105	REACTION 2	RE1	LABEL1
		RE2	LABEL2
		RE3	LABEL3
		PROBE P	LABEL4
			1112
1109	REACTION 15	RE36	LABEL3
		RE37	LABEL4
1100			1110
			1111

FIG.7

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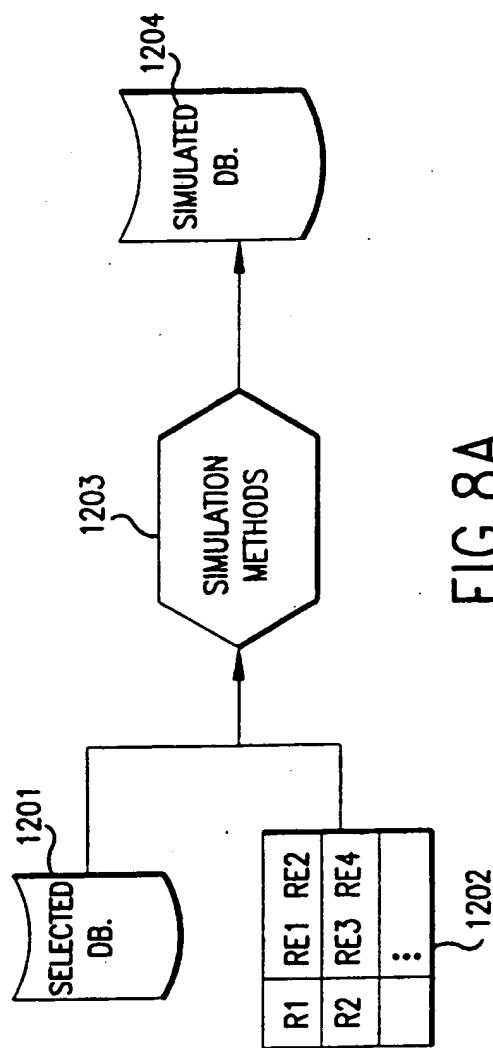


FIG.8A

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L	$(RE1, RE1)_{R1}$	1213	1214	$(RE1, RE2)_{R1}$	$(RE2, RE2)_{R2}$	$(RE3, RE3)_{R2}$	$(RE3, RE4)_{R2}$	\dots
\dots	\dots	1215						
\dots	52	$A01$				1216		
\dots	\dots							
1211	\dots	1218						
\dots	151	$T163$	\dots	1217		$A01,S003$		
\dots	\dots							
175				$A01,T163$				
\dots	\dots				1220			
\dots	222				$T163,Q012$			
\dots	\dots							
402							1219	
\dots	\dots							
532							$Q012,S003$	
\dots	\dots							
							1210	

FIG. 8B

SUBSTITUTE SHEET (RULE 26)

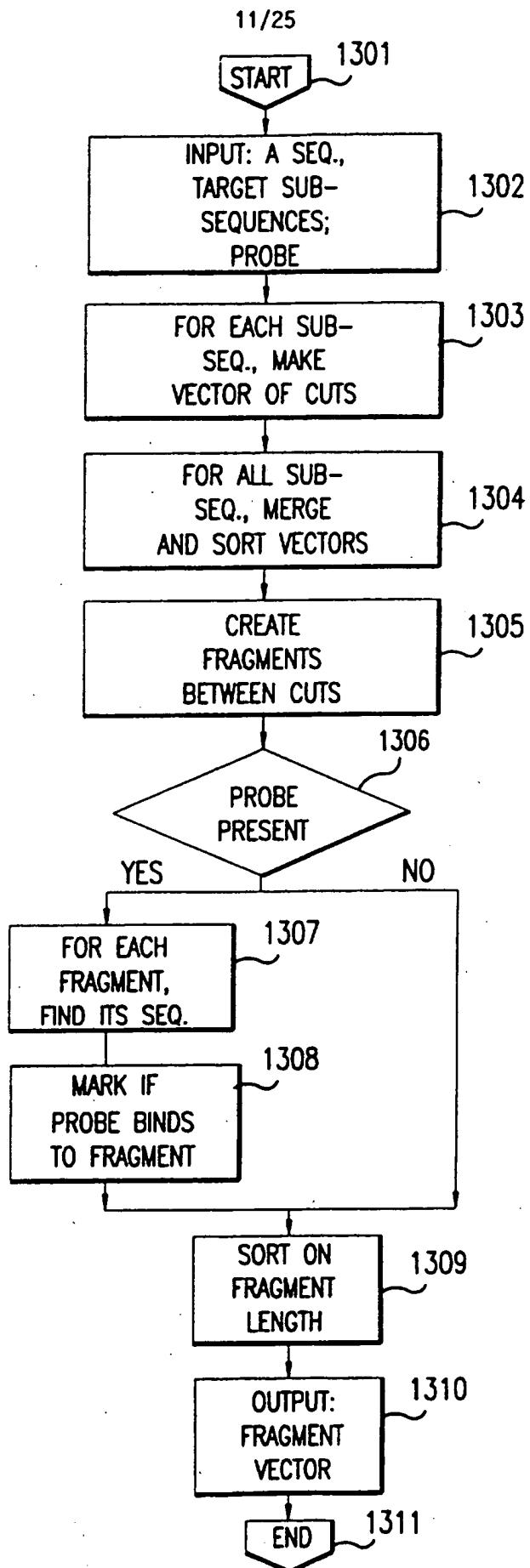


FIG.9

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1403

1401 ~ $v_1 = ([10,14]_{RE1}, [62,66]_{RE1}, [610,614]_{RE1})$

1402 ~ $v_2 = ([237,241]_{RE2}, [388,392]_{RE2})$

VECTORS OF CUTS

1404 1405 1406 1407

([10,14]_{RE1}, [62,66]_{RE1}, [237,241]_{RE2},
 $[388,392]_{RE2}, [610,614]_{RE1})$

MERGED AND SORTED VECTORS OF CUTS

FIG.10A

1408 1409

[RE1,RE1,*,52] [RE1,RE2,*,175] [RE2,RE2,*,151]

[RE1,RE2,*,222]

FRAGMENTS

FIG.10B

1410

[10,62] [62,237] [237,388] [388,610]

FRAGMENTS SEQUENCES

FIG.10C

SUBSTITUTE SHEET (RULE 26)

FIG.10D

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1411

([RE1,RE1,N,52] , [RE2,RE2,y,151] ,
 [RE1,RE2,N,175] , [RE1,RE2,N,222])

1412

SORTED FRAGMENT
 VECTOR

FIG.10E

↓

	[RE1,RE1]	[RE1,RE2]	[RE2,RE2]	
52	+A01			1412
151				+A01
175		+A01		
222		+A01		

DIGEST
 TABLE

FIG.10F

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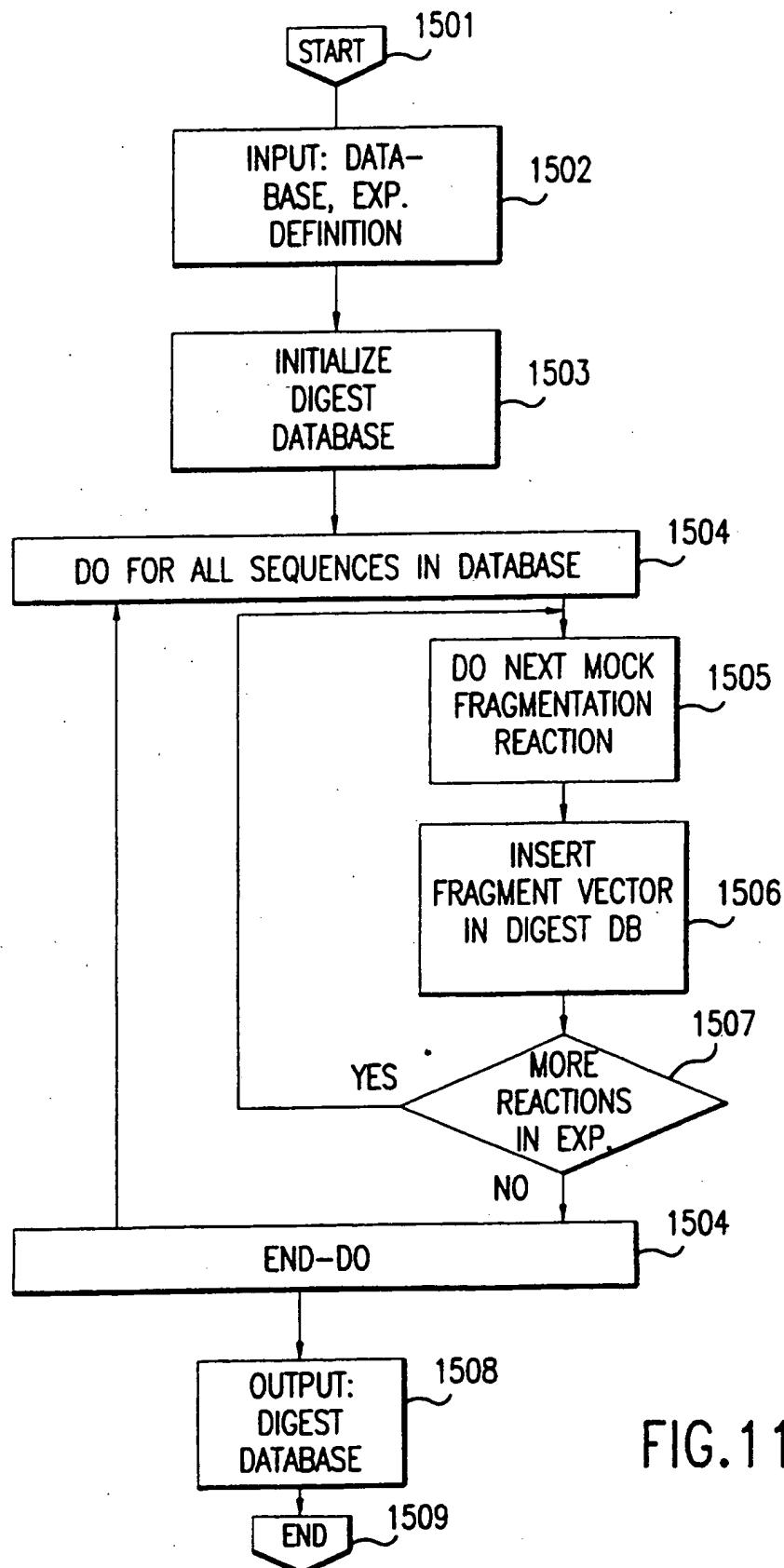
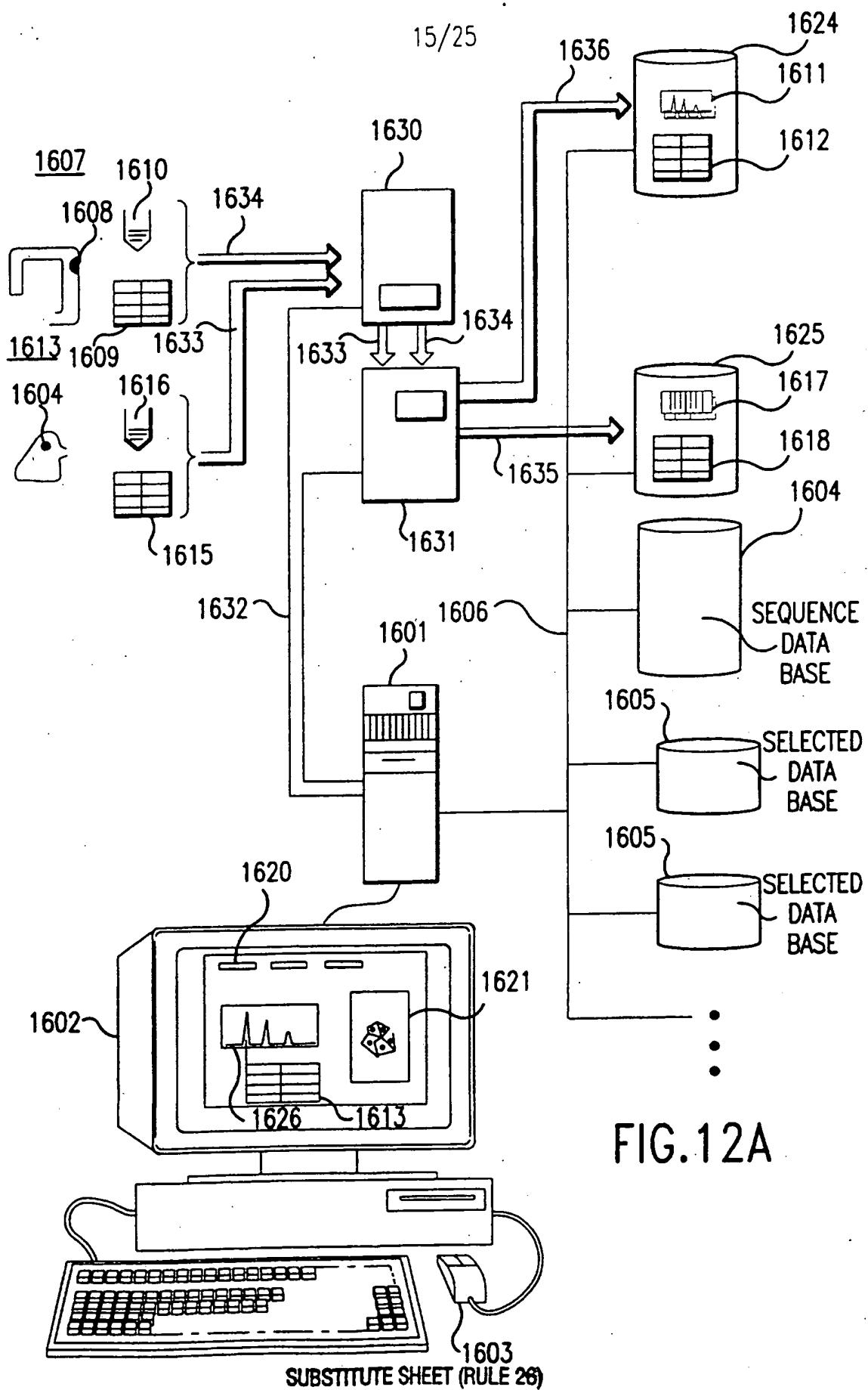


FIG.11



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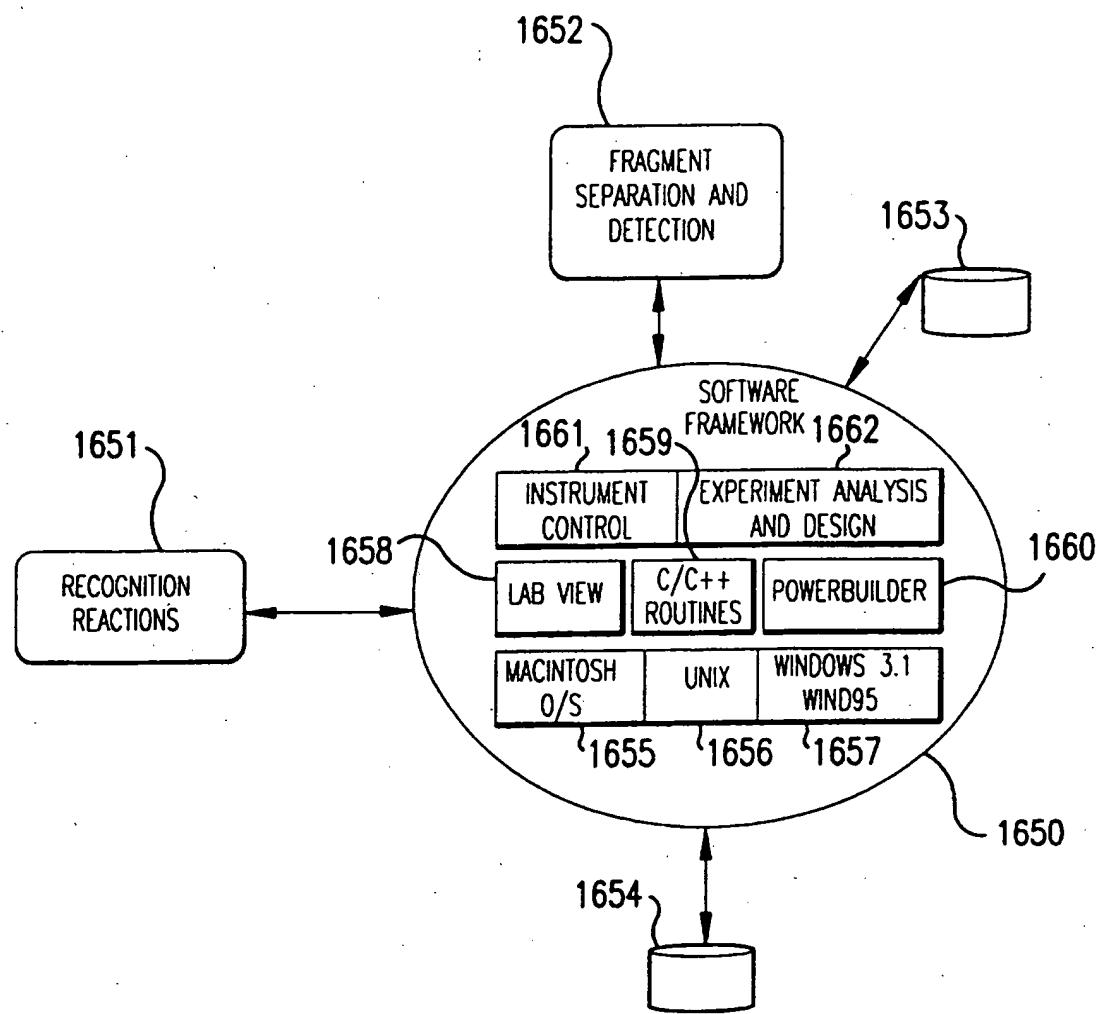


FIG.12B

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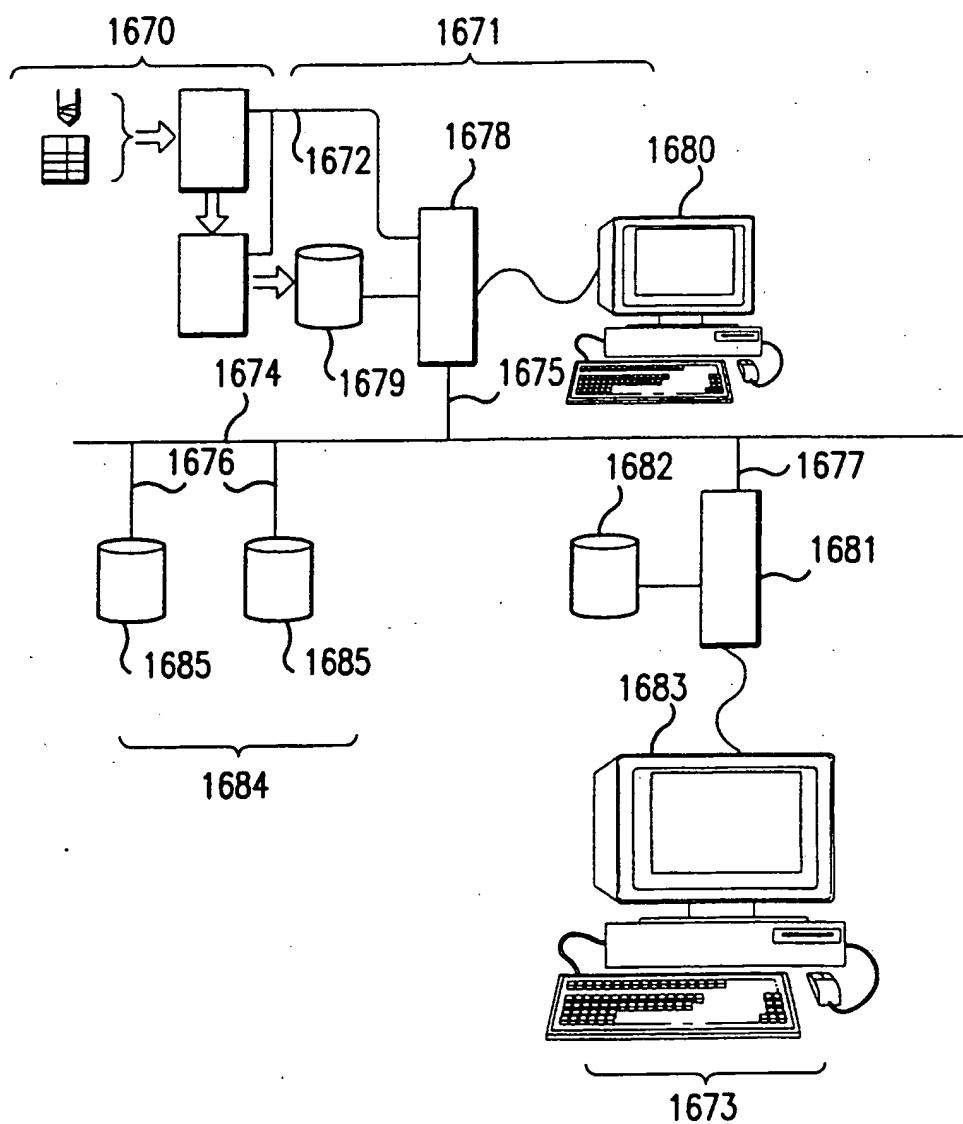


FIG.12C
SUBSTITUTE SHEET (RULE 28)

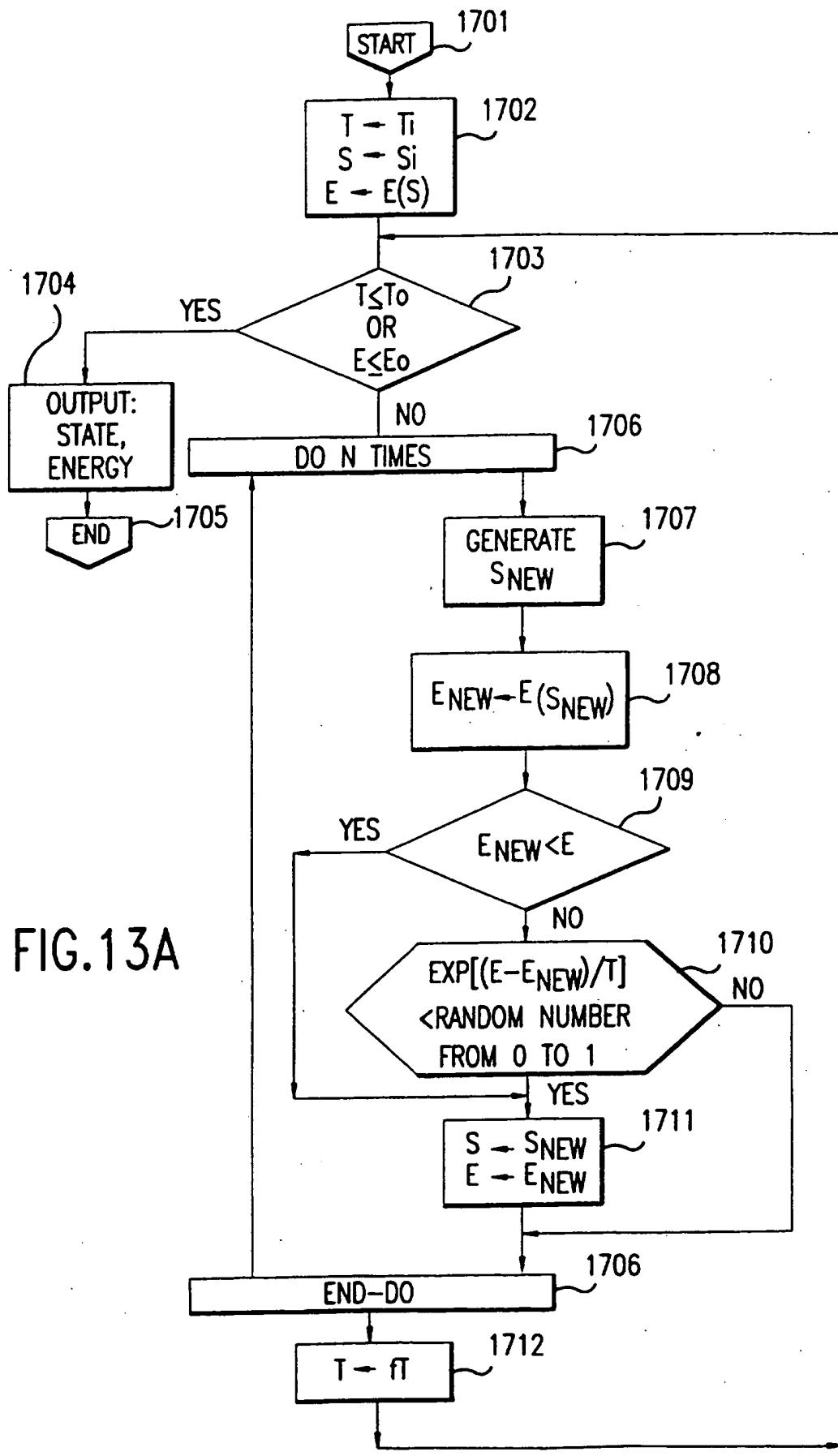


FIG.13A

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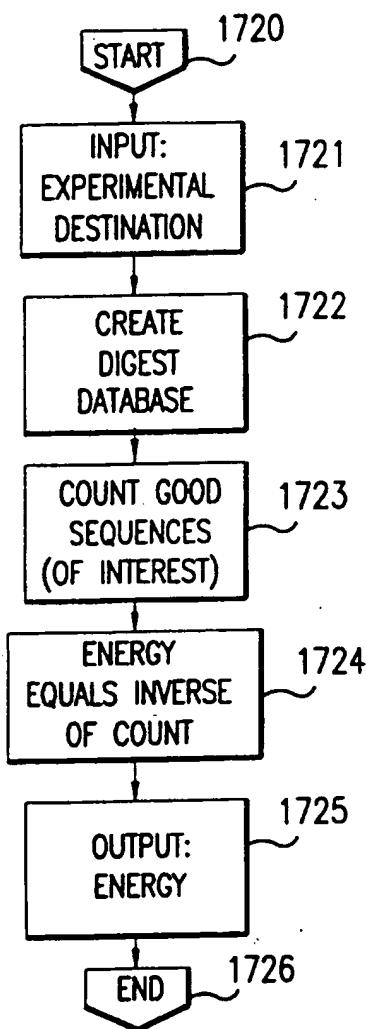


FIG.13B

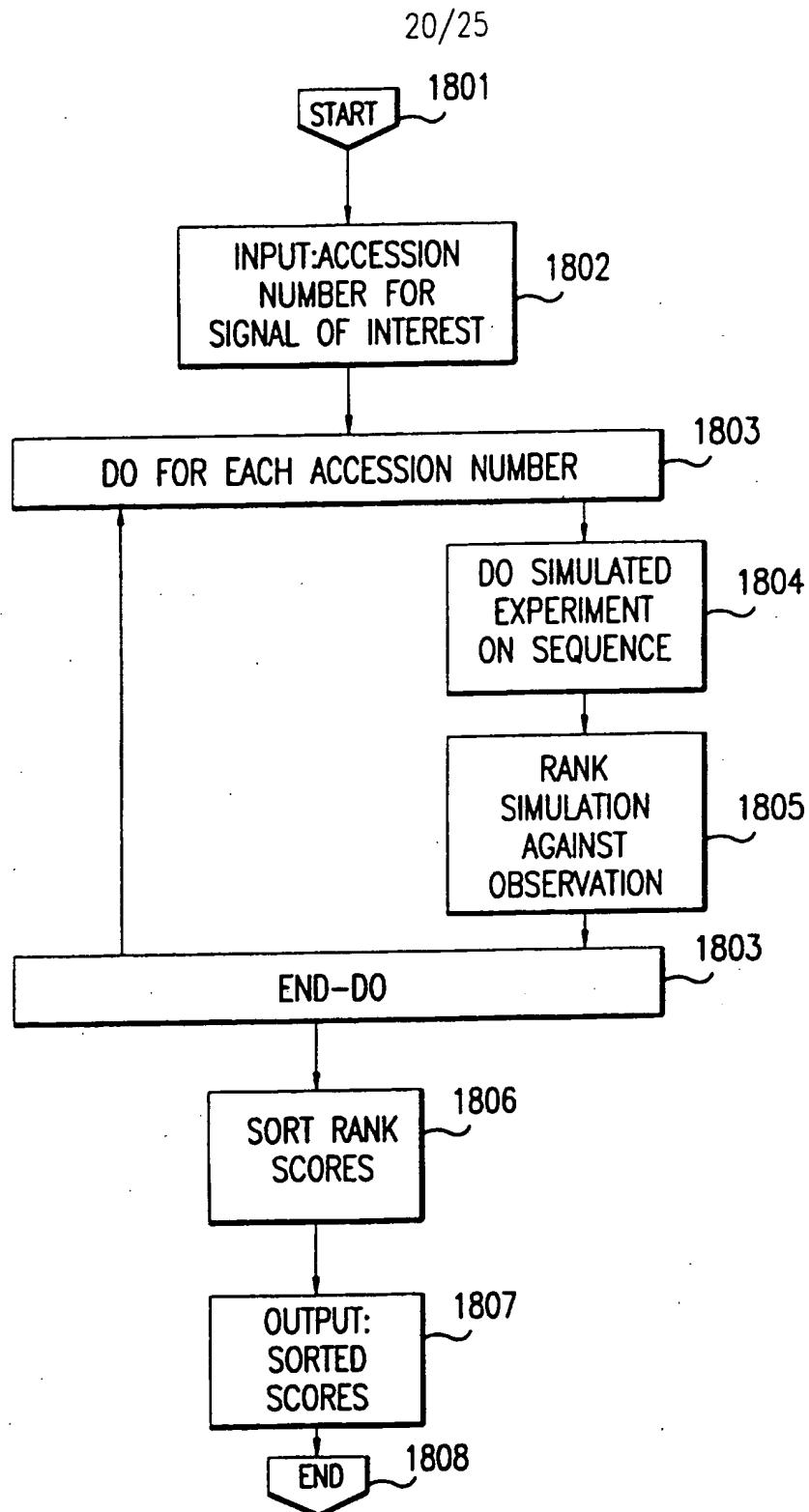


FIG.14
SUBSTITUTE SHEET (RULE 26)

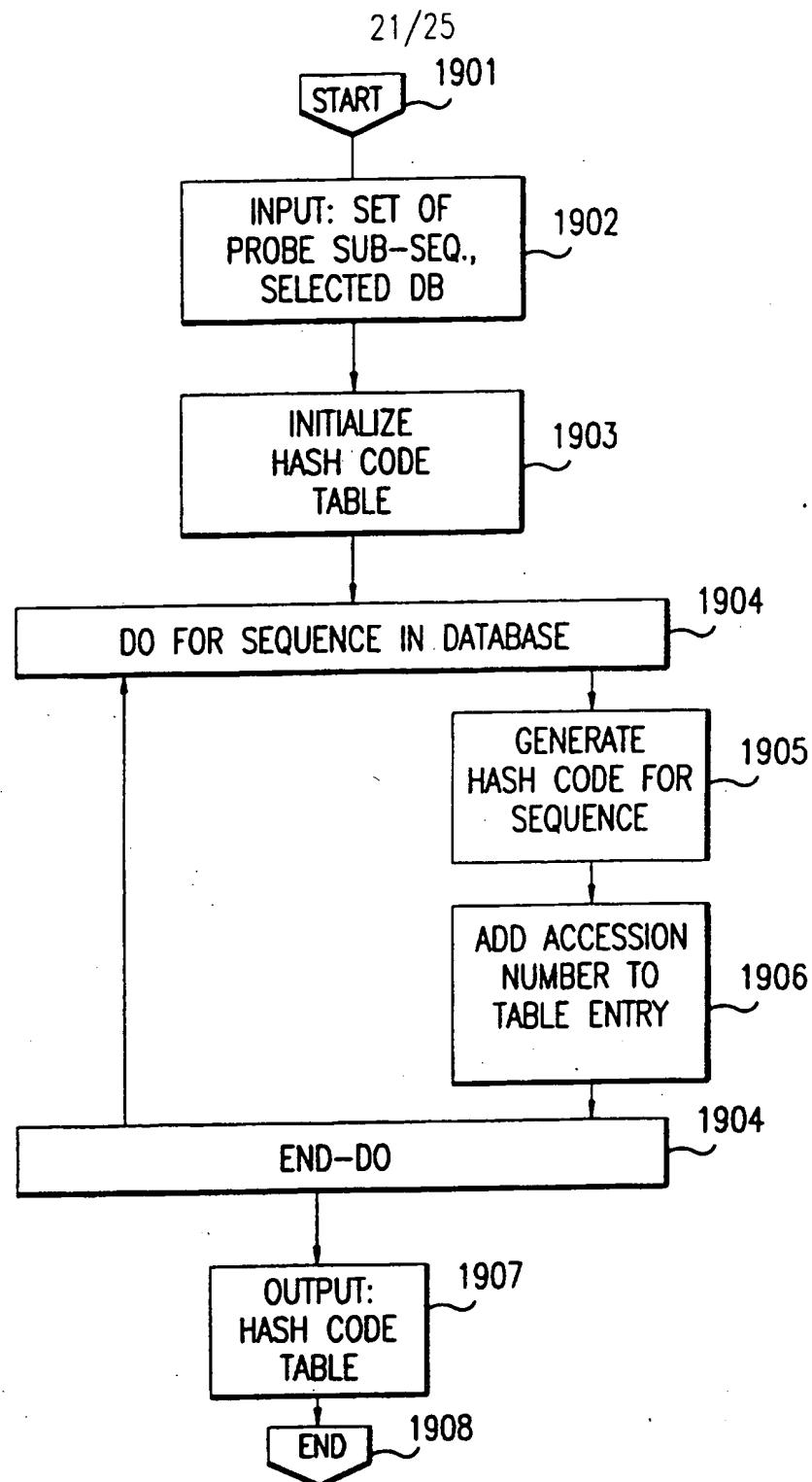


FIG.15

SUBSTITUTE SHEET (RULE 26)

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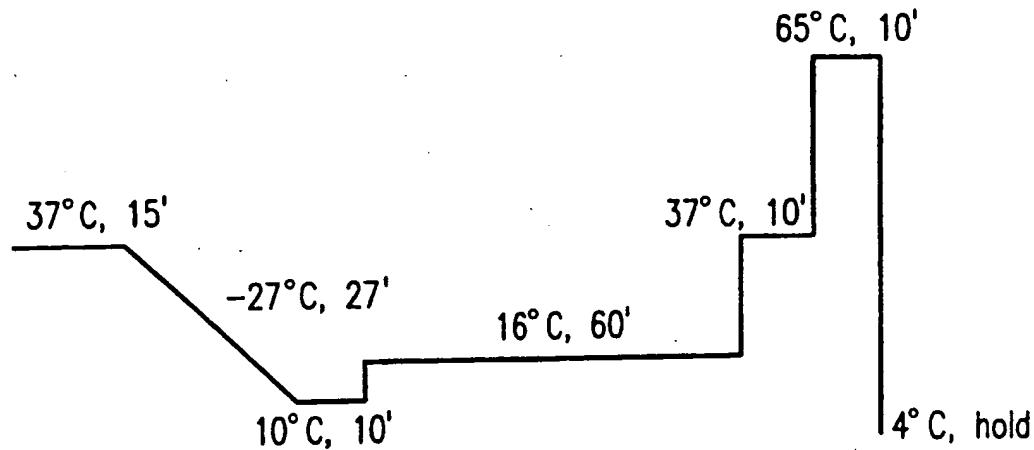


FIG.16A

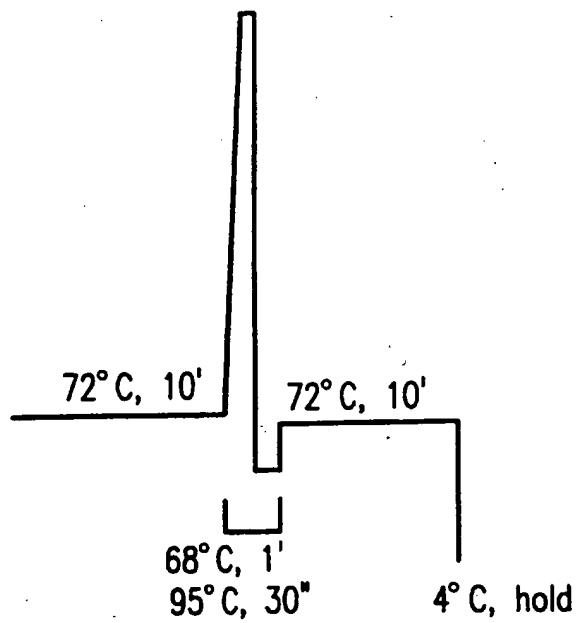


FIG.16B

SUBSTITUTE SHEET (RULE 26)

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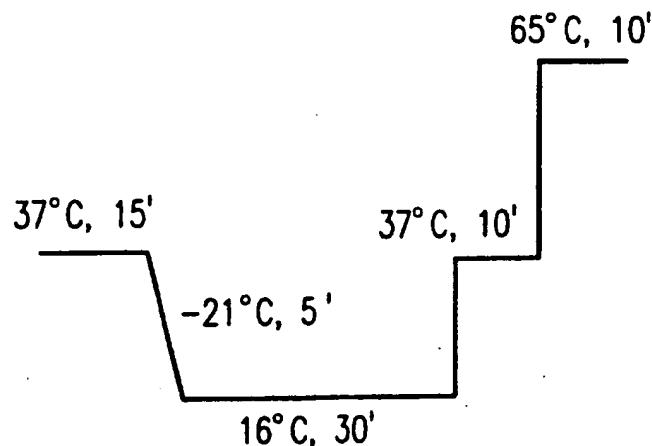
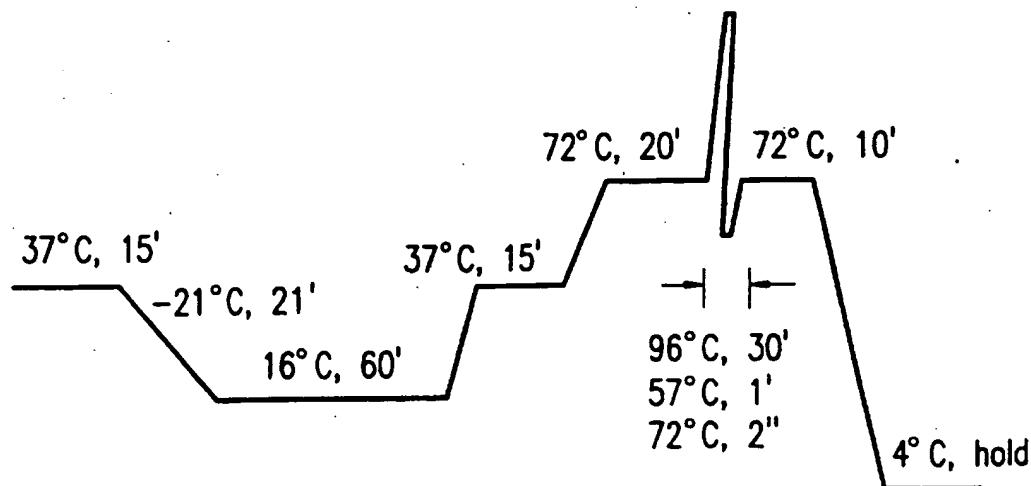


FIG. 16C

FIG. 16D
SUBSTITUTE SHEET (RULE 26)

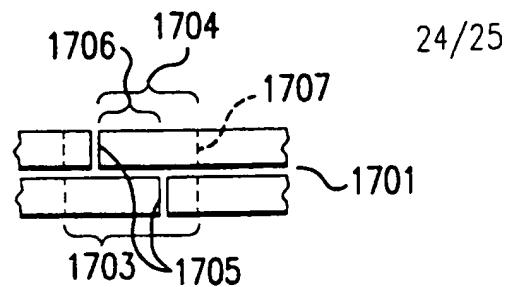


FIG. 17A

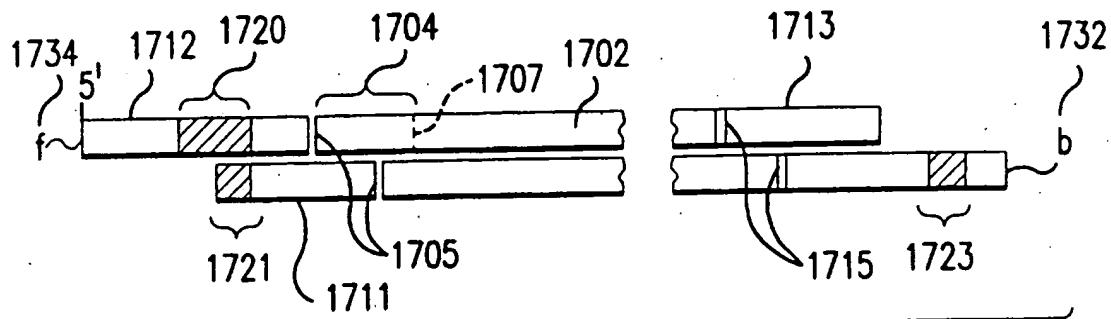


FIG. 17B

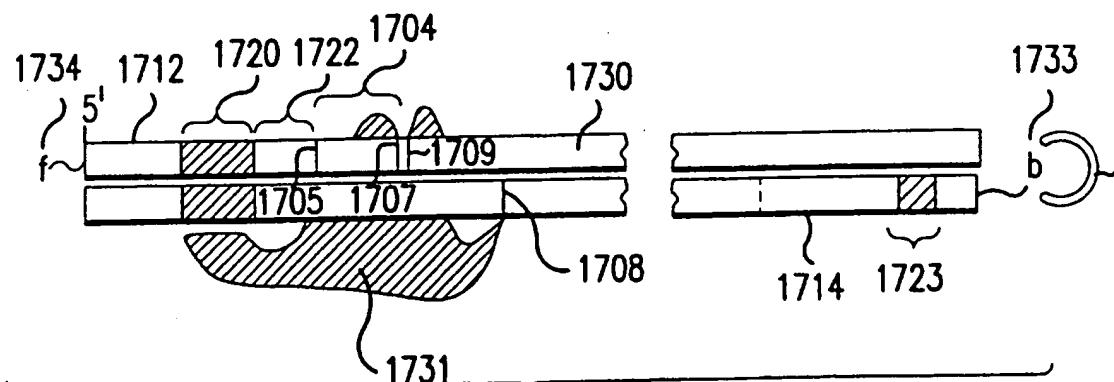


FIG. 17C

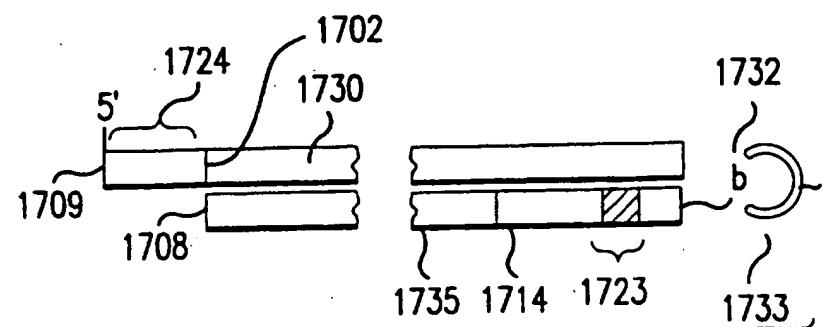


FIG. 17D

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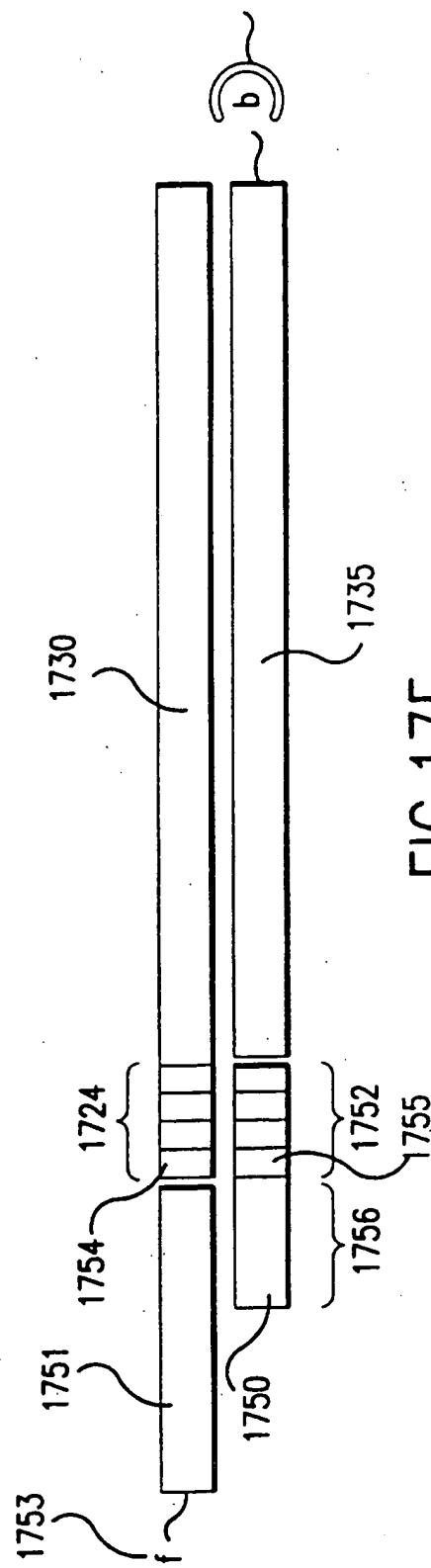


FIG. 17E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/17159

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.1, 91.2, 291; 422/ 82.01, 82.07; 536/ 23.1, 24.3, 24.33; 364/ 496, 499

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PEASE et al. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc. Natl. Acad. Sci. May 1994, Volume 91, pages 5022-5026, see entire document.	1-259
Y	MEIER-EWERT et al. An automated approach to generating expressed sequence catalogues. Nature. 28 January 1993, Volume 361, pages 375-376, see entire document.	1-259
Y	MAIER et al. Hybridisation techniques on gridded high density DNA and in situ colony filters based on fluorescence detection. Nucleic Acids Research. 1994, Volume 22, Number 16. pages 3423-2424, see entire document.	1-259

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	*&*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
22 DECEMBER 1996	13 FEB 1997

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIANNE REES Telephone No. (703) 308-0196
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/17159

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ITO et al. Fluorescent differential display: arbitrarily primed RT-PCR fingerprinting on an automated DNA sequencer. FEBS Letters. 1994, Volume 351, pages 231-236, see entire document.	1-259
Y	KATO, K. Description of the entire mRNA population by a 3' end cDNA fragment generated by class IIS restriction enzymes. Nucleic Acids Research. 17 October 1995, Volume 23, Number 18, pages 3685-3690, see entire document.	1-259
Y	US 5,366,877 A (KEITH ET AL) 22 November 1994, see abstract and Figure 1.	1-259
Y	WO 95/21944 A1 (SMITHKLINE BEECHAM CORPORATION) 17 August 1995, see abstract.	137-141
Y	EIGEN et al. Sorting single molecules: Application to diagnostics and evolutionary biotechnology. Proc. Natl. Acad. Sci. USA. June 1994, Volume 91, pages 5740-5747, especially 5742-5744.	1-259
Y	JU et al. Fluorescence energy transfer dye-labeled primers for DNA sequencing and analysis. Proc. Natl. Acad. Sci. USA. May 1995, Volume 92, pages 4347-4351, see abstract.	1-259
Y	GUO et al. Direct Fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. Nucleic Acids Research. 1994. Volume 22, Number 24, pages 5456-5465, see entire document.	1-259

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/17159

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/68, 170; C12P 19/34; C12M 1/34; G01N 27/00, 21/64; C07H 21/02, 21/04; G06F 17/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 5, 91.1, 91.2, 291; 422/ 82.01, 82.07; 536/ 23.1, 24.3, 24.33; 364/ 496, 499

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, BIOBUSINESS, CABA, CANCERLIT, CAPLUS, EMBASE, IFIPAT, JAPIO, MEDLINE, USPATFULL, WPIDS

Search terms: gene expression, sequence identification, classifying, quantifying, restriction enzyme digestion, Type IIS restriction enzymes, labels, signals, fluorescent, arrays, SBH, sequencing by hybridization, mismatch detection, automation and sequencing, differential display, arrayed cDNA libraries, adaptor or linker sequences, base calling, sequencing databases, computer programs and SBH analysis.